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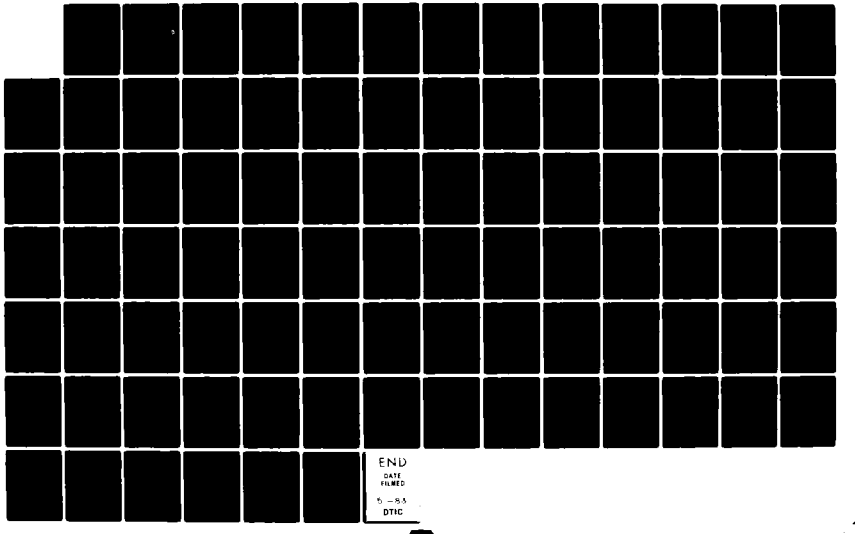

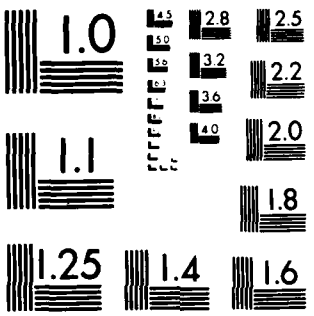

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NEUROPHYSIOLOGICAL STUDY OF VECTOR RESPONSES TO REPELLENTS

ANNUAL AND FINAL REPORT

Edward E. Davis

August 1980

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U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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Nectar and blood feeding and oviposition, which are important to the survival of the mosquito, depend heavily on the detection of airborne chemical signals. Our research objective, therefore, was to gain a better understanding of the basic mechanisms of chemoreception and the action of repellents on the sensory system so that a more rational approach would be possible in the development of methods to prevent disease transmission by these organisms.

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The definition of insect repellents has been based on the practical consideration that they prevent biting by the female. This led to the notion that repellents are a single class of compounds having a single mode of action.

The results of our investigations led us to postulate four modes of action of repellents on the peripheral chemosensory neurons.

(1) Some repellents interfere directly with the detection of host attractants and thereby prevent host-seeking behavior (deet and 612 inhibit the detection of LA by the antennal LA-excited neurons).

(2) Some repellents elicit responses from sensilla that mediate a behavior other than host-seeking and thus direct that behavior instead of host-seeking.

(3) Some repellents activate receptors responsible for detecting noxious substances.

(4) High concentrations of some repellents saturate the sensory organs, resulting in the inhibition of host-seeking behavior.

Changes in the physiologic state of A. aegypti that are induced by the blood meal correspond in time with the inhibition of host-seeking behavior. In addition, about 48 h after a blood meal, we observed an enhanced sensitivity of the short, pointed sensilla to the oviposition attractant methyl butyrate. This corresponds to the time of maturation of the ova and onset of oviposition. Following oviposition, the sensitivity of the LA-sensitive neuron returns to pre-blood-meal levels. We suggest that this could be mechanism for the selection and modification of the overt behavior pattern of female mosquitoes.

SUMMARY

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CONTENTS

INTRODUCTION

The nature of military operations during an armed conflict usually prevents the initiation of vector control measures before deployment of troops in the area. As a result, personnel may be exposed to a high risk of infection from vector organisms carrying such diseases as malaria, yellow fever, dengue, and encephalitis. For protection against these vector organisms, military personnel topically apply chemical repellents and wear special repellent-treated outer clothing. However, the most commonly used chemical repellent, deet, has several drawbacks associated with its use, and investigators are seeking newer formulations and/or new repellent substances that would be superior to existing ones.

After several years of empirical searching, a suitable replacement for the existing repellent materials has not been found. One problem encountered is that chemists design new repellent compounds without any knowledge of how these substances act on the sensory system of the target organisms. These efforts could be more productive if we had greater knowledge about the sensory basis for the behavior patterns of vector organisms and used this information to direct development efforts along more rational lines.

Recent studies of the neurophysiologic activity of the peripheral receptor organs of mosquitoes provided us with a basic understanding of some of the fundamental sensory processes underlying the behavior of these vectors. For example, our finding that repellents block the response of a certain chemoreceptor neuron to lactic acid--normally an attractive substance--has led to the development of at least one potential repellent based on the structure of lactic acid.

We also thought it important to elucidate (1) the sensory-behavior mechanisms of disease vector organisms and (2) how they are influenced by the various physiologic states of the organism. Complex integrated

behavior has been thought to result principally from processes in the central nervous system; the peripheral sensory organs supplied information about the environment on which the CNS would operate. From data obtained in the project reported herein, we now know that the peripheral sensory organs are affected by the physiologic condition of the organism after a blood meal and that these changes may not only modify the information transmitted to the CNS, but may actually cause the selection of one behavior pattern instead of another.

Another major problem in the development of new repellent materials has been that we lacked knowledge of how different species of mosquitoes respond to important behavioral stimuli such as host-related substances, oviposition attractants, and insect repellents. We attempted to gain an insight into this problem by comparing the responsiveness of the peripheral chemosensory elements of several species of mosquitoes (Aedes aegypti, A. triseriatus, Culex tarsalis, and Anopheles stephensi). In the process, we encountered difficulty in defining what an insect repellent was and what it did to the behavior of a mosquito. Insect repellents are defined on a practical basis--i.e., prevention of biting--not on a strictly behavioral or chemical basis. This makes the investigation of how "repellents" act extremely difficult. However, we did find evidence for some potential modes of action for existing substances categorized as "repellents."

We hope that our investigations of the sensory basis of the behavior patterns of the various vector organisms and of the relationship between the physiologic condition of the organisms and the generation and transmission of sensory information will be useful in the development of new and improved materials for the regulation and control of vectors of medical and military importance.

OBJECTIVES

The goals of the proposed research were to gain a better understanding of the role of the peripheral sensory system in modulating the behavior of disease vector organisms and to provide information that may be useful in the eventual regulation and control of these organisms.

Specifically, we proposed to:

(1) Compare the responses of antennal chemoreceptors of different species and strains of mosquitoes and other arthropod vectors that are sensitive to chemical repellents and to other behaviorally active stimuli.

(2) Investigate how the various physiologic states of an organism affect the ability of its peripheral receptor organs to perceive and transmit sensory information to the CNS.

(3) Investigate the responses of contact chemoreceptor organs on the labella and tarsi of mosquitoes that are associated with the feeding behaviors--nectar and blood feeding--and the effects of chemical repellents on these responses.

(4) Correlate the neurophysiologic results with the morphology and the behavior of vector organisms.

METHODS

General Electrophysiologic Procedures

The electrophysiologic and stimulus-generating equipment and techniques used in this study have been described in detail (Davis and Sokolove, 1975, 1976, and in the Appendix). These techniques, with some modifications, are briefly described.

Experimental Setup

Female mosquitoes at least three days postemergence were anesthetized with CO_2 and placed supinely on a brass mount, with their wings secured with water-soluble glue. The antennae were secured to double-stick Scotch tape. An uninsulated tungsten microelectrode (< 1-μ tip diameter) was inserted into the hemolymph space at the tip of the antenna and connected to ground. A similar recording electrode was inserted through the cuticle at the base of a sensillum and connected to a Grass P-15 preamplifier to detect and amplify the nerve spikes. The resulting signal was filtered in a band of 300 Hz to 2 KHz, displayed on a Tektronix 561A oscilloscope with a 3A74 preamplifier, and recorded on an Ampex SP-300 tape recorder. The nerve spikes were also routed to an F. Haer three-channel amplitude discriminator, the output of which is displayed on the oscilloscope, recorded on magnetic tape, and connected to a spike frequency converter for plotting the instantaneous (spike-to-spike) frequencies of up to three individual neurons on a Brush 240 recorder.

Stimulus Delivery

Olfactory stimuli were generated by saturating a stream of synthetic air (79% N_2, 21% O_2) at constant temperature and pressure. The intensity of the stimulus was determined by the rate of airflow and the vapor pressure of the test substance. The flow rates were controlled by

individual flow meters and valves. Up to six different stimuli could be connected to a six-channel, solenoid-operated, manifold/switching device. The saturated airstreams were added to a carrier airstream (typically 50% RH, 28°C, and 1 liter/min flow rate) directed over the mosquito. Since none of the streams was interrupted by switching, absorption effects were minimized and confined to a replaceable glass nozzle. Each stimulus airstream could be switched individually or in combination, simultaneously or sequentially, or the airstreams could overlap.

To saturate an airstream by bubbling it through the test solution, we needed at least a 1-ml sample. When this minimum volume was not available, we applied smaller aliquots of the sample to small pieces of filter paper of uniform size and placed them in glass tubes, through which the airstream was passed, rather than in the gas bubbler flask. To determine the accuracy of our stimulus-intensity calculations, we periodically took samples of the various stimuli for analysis with a gas chromatograph-mass spectrometer.

A third, qualitative method of odor presentation that we used to screen up to 38 chemical stimuli on single chemoreceptor neurons was to saturate a small piece of filter paper with the test substance and place it in a 10-ml syringe. The air in the syringe was then manually expelled over the mosquito (Davis, 1976).

Our usual procedure was to first use the qualitative stimulus presentation to determine which substances produced responses in the sensillum being examined. Having thus identified which stimuli elicit responses that are of interest, we then used one of the more quantitative chemical stimulus presentation systems.

Experimental Design

Objective 1

To accomplish our first objective--to compare the neurophysiologic responses of the peripheral receptor organs of different species and strains of mosquitoes and other anthropod vectors to chemical repellents and other behaviorally important stimuli--we divided our protocol into two phases.

In Phase 1, we initially screened a broad range of chemical substances over the antennal chemosensory organs. The chemical substances were grouped according to the behavior with which they were most closely associated according to various sources in the literature. From these experiments, we attempted to (1) identify the specificity of different morphological types of sensilla of several species of mosquitoes and the chemical stimuli associated with different behaviors and (2) correlate this information with known differences in the behavioral patterns of the different mosquito species (Objective 4). The species selected for comparison were Aedes aegypti, A. triseriatus, Culex tarsalis, and Anopheles stephensi obtained from the University of California, Berkeley, CA.; University of Maine, Oronome; and LAIR, San Francisco, CA., respectively.

Phase 2 was to obtain information about the sensitivities of selected morphologic types of sensilla to specific chemical substances. This would allow the comparison of the quantitative differences in sensory information between mosquito species that might help explain any behavioral differences. To accomplish this phase, we selected chemical stimuli from Phase 1 that evoked the greatest response from a given sensilla type, and presented these stimuli in a graded-intensity series while noting the change in spike frequency at each stimulus intensity. The resulting data were graphed, and comparisons were made between homologous sensilla on the same or different species of mosquitoes. This method also permitted the study of the interactions of two chemical substances presented together--e.g., attractants and repellents.

Objective 2

Our second objective was to investigate how the various physiologic states of an organism affect the ability of its peripheral receptors to perceive and transmit sensory information to the CNS.

We chose to investigate the change in physiologic state that occurs after the female obtains a blood meal. This decision was based on the results of recent investigations by Klowden and Lea (1978, 1979) regarding the behavioral changes observed after a blood meal. They showed that

after the female feeds on a host, she becomes unresponsive to further host stimuli and will not engage in host-seeking behavior. Furthermore, a hemolymph-borne factor is responsible for this inhibition of host-seeking. We were interested in determining the site of action for this humoral factor--i.e., did it act on the peripheral sensory neurons or on some process in the CNS.

Other Objectives

We had originally planned to work on accomplishing the third objective during the second and third years of the proposed project. Funds for those years were not granted. Objective 4 was achieved in the course of accomplishing objectives 1 and 2.

RESULTS AND DISCUSSION

Objective 1

The results of the experiments conducted to accomplish Objective 1 provided some insight into how different species of mosquitoes detect different chemical substances that elicit important behaviors.

Table 1 summarizes our results for all species of mosquitoes according to behavior-evoking categories of the chemical stimulus and the types of antennal sensilla. Certain types of sensilla respond primarily to only one behavioral class of stimuli. For example the LA-sensitive neurons--both LA-excited and LA-inhibited--respond to host-related compounds but not to oviposition- or nectar-related stimuli. As the stimuli were presented at levels much higher than the mosquito might encounter in nature, the data only suggest which substances--e.g., those evoking strong responses or are of great interest (repellents)--should be examined at lower, more-physiologic intensities.

Thus, from the information in this table, we could select which type of sensilla has the highest probability for responding to substances within a behavioral catagory. Information such as this argues strongly for a labelled-line organization of information transmission rather than a cross-fiber pattern system. In a labelled-line system the peripheral sensory organs may actually select and modulate behavior patterns depending on which one(s) is (are) active or affected by the stimuli. In contrast, in a cross-fiber pattern system, the peripheral responses do not provide information along organized recognizable channels; their input must be taken *in toto* for filtering and integration in the CNS. In both systems, the overt behavioral response is the result of activity patterns in the motor neurons located centrally.

Table 1

SUMMARY OF THE QUALITATIVE RESPONSE CHARACTERISTICS OF THE DIFFERENT ANTENNAL SENSILLA OF FOUR SPECIES OF MOSQUITOES TO BEHAVIOR-RELATED CATEGORIES OF CHEMICAL STIMULI

Behavioral Category of Stimulus	Sensilla-Type: Sensilla Trichodea (Sensory hair) A1	A2-I (long blunt)	A2-II (short blunt)	A2 (short pointed)	Grooved-peg Sensilla LA-exc.	LA-inhib.
Host-Related	0	0	+	0	++	--
Oviposition	0	0	+/0/-[1]	++[2]	0	0
Repellents[3]	0	+/0[4]	++/0	+/0	+/0/-[5]	+/0/-
Plant-related (nectar)	+	+	++	0	0	0

- Primary response patterns of the chemosensory afferent nerves are indicated as excitatory (+), inhibitory (-), or no response (0); and whether strong (++ or --), or weak (+ or -), or no clear response pattern.

1. The reaction is not specific to compounds known to evoke behavioral responses in a given species.
2. The excitation is species specific.
3. The repellents are classified 'operationally' and not by the specific behavioral response they might evoke.
4. These are the results of the present study which are in conflict with earlier findings of strong excitation by Deet and 612 in this type sensilla (Davis and Rebert, 1972).
5. The effect of "repellents" seems to be different on different sensilla and between different substances classed as repellents.

The peripheral sensory system of the mosquito appears to be organized in a labelled-line manner. Sensory systems of other insects may not be organized in this way but rely on the recognition of patterns of activity generated by chemical stimuli across a large population of neurons whose specificity is more loosely defined. With the labelled-line system, it is easier to identify the initial components whose activity might be modified to control the insect's behavior. For example, the repellent, deet inhibits the response of the LA-excited neuron to the host attractant, LA (Davis and Sokolove, 1976).

We examined five of the six known physiologic types of sensilla in detail. The sixth, the long pointed sensory hair (A1), was not routinely sampled because apparently is associated with nectar-feeding (Lacher, 1967), a behavior of secondary interest to this study. We will discuss the responses of the various sensilla in relation to certain behavior patterns of the four species of female mosquitoes.

Oviposition-related stimuli. From Table 1, we can see that chemosensory neurons from two sensilla types respond to stimuli associated with oviposition behavior--the short pointed and the short blunt sensory hairs. Table 2 shows the number of sensilla of each type that responded to each chemical stimulus out of the total number of that sensilla type to which each stimulus was presented. Table 3 summarizes this information for ease of comparison. Perry and Fay (1967) found that gravid A. aegypti were attracted by methyl and ethyl esters of short chain fatty acids--e.g., methyl butyrate and ethyl propionate. Electrophysiologically, neurons of the short pointed sensilla respond strongly to these compounds but those of the short blunt sensilla are unresponsive. Both types of sensory neurons of the other three species gave basically no response (considering that the compounds were presented neat). This suggests that these compounds are highly specific to A. aegypti and that the neurons of the short pointed sensilla are sufficient to encode this information. Although A. aegypti sensilla responded to other compounds presented neat, when these other stimuli were presented at physiologic levels the chemosensory neurons were unresponsive except to the fatty acid esters and to 4-methylcyclohexanol (4-McH).

Table 2. Responses of A2-type sensilla trichodea of four species of mosquitoes to ovi-position-site-related chemical stimuli (all stimuli presented neat via syringe)

Species	Stimuli	Sensilla Responses: Short Pointed Resp.	Short Pointed No./N*	Long Blunt (A2-I) Resp.	Long Blunt (A2-I) No./N	Short Blunt (A2-II) Resp.	Short Blunt (A2-II) No./N
Aedes aegypti	Ethyl lactate	++	38/41	++	23/30	+/-	6/9
	2-Butoxy ethanol	++	29/35	++	27/29	++	6/7
	p-Cresol	0	28/31	0	26/26	++	7/9
	o-Cresol	0	13/19	0	21/21	++	9/10
	m-Cresol	0	11/14	0	18/18	+	5/7
	Ethyl propionate	++	7/24	0	17/18	0	4/5
	Methyl propionate	+	7/17	0	18/21	0	3/5
	Methyl butyrate	++	20/23	0	7/8	0	2/3
	Ethyl acetate	+	7/15			0	2/3
	4-Methylcyclohexanol	++	21/22	0	1/1	0	3/5
A. tinseriatus	Ethyl lactate	++	18/20	+	3/5	0	2/3
	2-Butoxy ethanol	++	9/15	++	3/5	+/-	2/3
	p-Cresol	0	8/15	0	2/3	++	7/7
	o-Cresol	+	5/12	0	4/5	++	5/6
	m-Cresol	+	5/13	0	3/3	+	4/6
	Ethyl propionate	0	9/10	+	1/2	0	2/2
	Methyl propionate	0	3/4	0	2/3	0	2/2
	Methyl butyrate	++	2/3	0	2/3		
	Ethyl acetate	0	3/5	0	3/5		
	4-Methylcyclohexanol	++	12/13	0	3/3	0	2/4
Anopheles stephensi	Ethyl lactate	++	8/8	++	3/3	++	4/5
	2-Butoxy ethanol	++	8/8	++	3/3	++	4/5
	p-Cresol	++	7/8	0	3/3	++	5/5
	o-Cresol	++	5/6	0	2/3	++	4/5
	m-Cresol	++	5/5	0	2/3	+	5/5
	Ethyl propionate	0	4/5	0	2/2	0	5/5
	Methyl propionate	0	3/4	0	3/3	0	1/1
	Methyl butyrate	0	2/3	0	2/3	0	1/1
Culex tarsalis	Ethyl lactate	++	8/9	++	2/3	--	5/12
	2-Butoxy ethanol	++	7/9	++	3/3	++	10/12
	p-Cresol	0	6/8	0	1/1	+	10/10
	o-Cresol	0	6/6	0	3/3	++	11/13
	m-Cresol	0	5/5	0	2/3	++	7/13
	Ethyl propionate	0	6/6			+	3/7
	Methyl propionate	0	4/5	0	1/1	0	6/9
	Methyl butyrate	0	2/2	0	1/1	+	3/4
	Ethyl acetate	++	2/3	+	1/2	0	3/4
	4-Methylcyclohexanol	++	4/5	++	1/1	+/-	6/8

*Number responding as indicated out of total number tested.

Table 3. Comparison of Responses from Two Types of Sensilla on Four Species of Mosquitoes to Oviposition Site Attractants

Sensilla Type:	Short pointed					Short blunt			
Species: / Compounds[1]	*A. aegypti*	*A. triseriatus*	*C. tarsalis*	*An. stephensi*	....	*A. aegypti*	*A. triseriatus*	*C. tarsalis*	*An. stephensi*
Ethyl-lactate	++[2]	++	++	++		+/-	0	--	++
2-butoxyethanol	++	++	++	++		++	+/-	++	++
fatty acid esters	++	+/0	0	0		0	0	+/0	0
p-cresol	0	0	0	++		++	++	+	++
O-, m-cresol	0	+	0	++		++	++	++	++
4-methyl-cyclohexanol	++	++	++	n.t.		0	0	+/-	n.t.
butyric acid	--	--	0	--	....	+	0	+/-	0

Note: 1-reference: EL, O-, m-, p-cresol, 4-MCH: M. Bentley, personal comm. (1979)
BOE: T. Ikeshoji (1968)
FAE: Perry and Fay (1967)

2-reference: Strong response (++ and --); weak to moderate response (+ and -); mixed response (+/0 and +/-); and no response (0) in greatest number of cases tested.

n.t. = not tested.

Bentley and co-workers reported finding small quantities of ethyllactate and p-cresol in larval and treehole water that would attract gravid A. triseriatus in the laboratory; Ø- and m-cresol were also present but acted as oviposition stimulants rather than as attractants. Neurons of the short pointed sensilla of A. triseriatus were strongly excited by ethyllactate and by 4-McH (an analog of p-cresol), whereas neurons of the short blunt sensilla were strongly excited by the cresol compounds. p-Cresol was most effective in eliciting both a behavioral and electrophysiological response for neurons of the short blunt sensilla (Fig. 1). As p-cresol alone is a strong oviposition attractant, excitation in the neurons of the short blunt sensilla in gravid A. triseriatus appear to be sufficient to encode oviposition attractants. Bentley tested 4-McH as part of a series of compounds to establish the structure-activity relationship of p-cresol in behavioral bioassay, and found that 4-McH, was also active. However, when we tested 4-McH and p-cresol, we found that 4-McH acted on the short pointed sensilla, while p-cresol reacted with the short blunt sensilla. Furthermore, trans-4-McH, which bears the greatest structural similarity to p-cresol, is less effective than cis-4-McH in evoking both an excitation of the neurons in the short pointed sensillum (Fig. 2) and behavioral attraction to an oviposition site. Thus, although 4-McH may have certain structural similarities with p-cresol, it does not react with the same receptor system. These results suggest that input from the short sharp sensilla may also indicate oviposition behavior in A. triseriatus. Examining the dose-response relationships of A. triseriatus and A. aegypti to 4-McH we find that A. triseriatus is more sensitive to 4-McH than A. aegypti--a finding one might expect given that of A. triseriatus to 4-McH. This indicates that extreme caution should be exercised when attempting to establish chemical structure-activity relationships based on the behavior of the organism.

The picture for C. tarsalis and A. stephensi is not as complete. Although the short sensory hairs of females of these two species respond with patterns differing from those of each other and the other two species, the stimuli that attract these species to an oviposition site with a high degree of specificity have not yet been identified. Ikeshoji (1968) found a substance in a rice-water infusion that attracted C. pipiens.

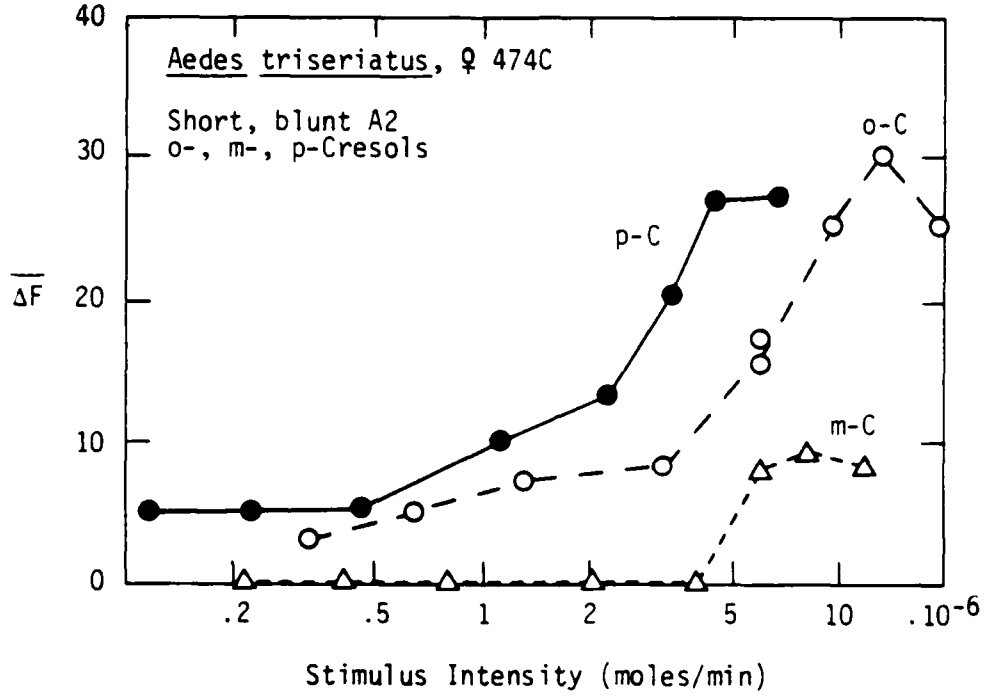


Fig. 1 Stimulus Intensity - Response function for neurons in a short, blunt A2 sensillum to O-, m, p-cresols.

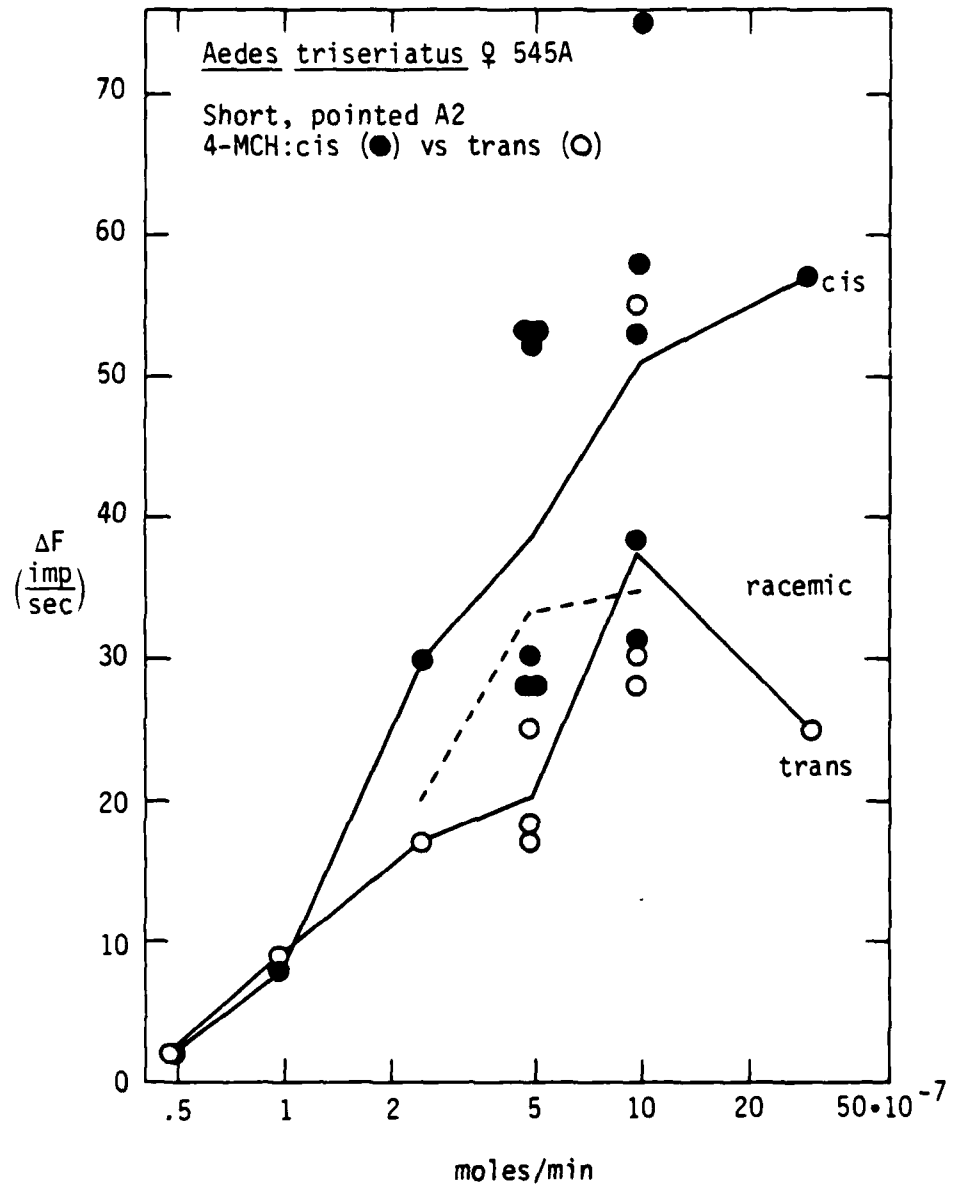


Fig. 2 Stimulus Intensity - Response functions for neurons within a short, pointed A2 sensillum to the isomers of 4-methyl cyclohexanol (4-McH).

He tentatively identified it as 2-butoxyethanol, but bioassay indicated that the pure substance was only weakly attractive.

Recent work reported by Hwang et al. (1980) indicated that gravid C. quinquefasciatus females were repelled from potential oviposition sites by the presence of small quantities of butyric acid. Therefore, we presented this substance to the oviposition-attractant-sensitive neurons and found that in three of the four species tested butyric acid inhibited the neurons of the short pointed sensilla and evoked weak or no responses in neurons of the short blunt sensilla. In the species most closely related to C. quinquefasciatus, C. tarsalis, neurons of the short pointed sensilla did not respond to butyric acid. However, a compound does not necessarily have to generate a direct response to exert an effect. It may block the capacity of the sensory neurons from responding to other attractive stimuli, as the repellent, deet, does on the LA-excited neurons. The action of butyric acid in antennal chemosensory neurons should be investigated to determine the site of and mode of action for repelling gravid female Culex mosquitoes from oviposition sites.

Host-related stimuli. The LA-sensitive neurons of the antennal grooved-peg sensilla of the four species of female mosquitoes examined exhibited no detectable difference in their response characteristics to the range of host-related compounds tested (Tables 4 and 5). This is not unexpected, because the substances tested were not specific to any of the preferred host organisms of the four mosquito species, and the LA-sensitive neurons are relatively specific for LA. For there to be strong interspecific differences, the stimuli would have to include substances unique to the preferred hosts of each species, and even then, the differences might be subtle, quantitative differences across the sensory neurons of several sensitive-types--i.e., a cross-fiber pattern for specific host recognition.

Table 4. Qualitative responses of the LA-excited neuron in the antennal groove peg (A3) sensilla species of mosquitos (♀♀) to chemical stimuli presented neat

Stimuli:	Sensilla Responses/each species							
	Aedes aegypti		Aedes triseriatus		Culex tarsalis		Anopheles stephensi	
	Resp.	No./N	Resp.	No./N	Resp.	No./N	Resp.	No./N
A. Host related and their analogs								
Lactic acid	++	38/38	++	8/8	+	2/2	++	5/5
Glyceric acid	++	4/9						
2-Br-propionic acid	+	1/1	+	3/3				
2-Cl-propionic acid	++	7/9	++	3/4	+/-	1/1	+	3/3
Butyric acid	+/-	5/5	+	1/2	-	1/1	0	1/1
Isobutyric acid	+/-	4/7	+/-	3/4			+/-	2/2
2-OH-isobutyric acid	+	2/4	0	2/2				
2200-10	0	4/5					+	1/2
Acetic acid	+	3/3						
Water vapor	0	1/1	0	3/3				
Ammonia	++	7/7	++	3/4	+	1/1		
B. Repellent								
Deet	0	14/16	++	4/6	++	1/1	0	2/2
Indalone	+	1/2	0	1/1			0	1/1
612	0	6/11	+	2/4	+	1/1	0	1/1
SRI-C 6	+	2/4	++	2/2	0	1/1	0	1/1
2504-5	0	5/5	0	1/1				
Citronellol	+	1/2						
Geraniol	+	1/2	0	1/1				
Napthalene	0	3/3					0	1/1
Juglone	++	1/2						
Vanillin	--	1/2	0	1/1				

+, ++, increased spile frequency; 0, no change in spike frequency: -, --, decreased spike frequency; #, no. of sensilla showing +, 0 or - response: N, total no. of sensilla tested.

SRI-C6: n-hexyl-triethyleneglycol-mono-ether
2200-10: n-butyl-morpholine-2,3-dione
2504-5: 1-carboxymethyl-6-pentyl cyclohexene

Table 5. Qualitative responses of the LA-inhibited neuron in the groove peg (A3) sensilla to chemical stimuli presented neat

Stimuli:	Sensilla Responses: Aedes aegypti Resp.	Aedes aegypti No./N	Aedes triseriatus Resp.	Aedes triseriatus No./N	Culex tarsalis Resp.	Culex tarsalis No./N	Anopheles stephensi Resp.	Anopheles stephensi No./N
A. Host related and their analogs								
Lactic acid	--	39/43	--	10/10	-	3/3	--	7/7
Glyceric acid	--	3/4	0	2/3				
2-Br-propionic acid	0	4/5	-	3/3	0	1/1	-	2/3
2-Cl-propionic acid	-	10/17	--	4/4	-	1/2	--	5/6
Butyric acid	++	12/19	--	3/4	0	1/1	-	2/2
Isobutyric acid	++	6/12	--	6/7	+	1/1	--	5/5
2-OH-isobutyric acid	-	2/3	0	3/4			-	1/2
2200-10	0	3/3	0	2/3			0	2/3
Acetic acid	--	4/6	--	2/2			-	1/2
Water vapor			-	1/1			0	1/1
Ammonia	++	19/19	++	5/6			+	1/1
B. Repellent								
Deet	0	8/15	0	3/6	not tested		-	3/4
Indalone	0	3/4	-	1/2			-	2/3
612	0/-	6/7	0	3/4			--	3/5
SRI-C 6	0	4/7	-	3/5			-	3/5
2504-5	0	3/4	0	2/2			0	3/3
Citronellol	0	4/6	0	3/3			-	1/2
Geraniol	0	3/3	-	1/2			-	1/2
Napthalene	0	2/2	0	3/3			0	1/1
Juglone	+	1/1	+	1/2			++	1/1
Vanillin	++	2/4	0	1/1				

+, ++, increased spike frequency: 0, no change in spike frequency; -, --, decreased spike frequency; #, no. of sensilla showing +, 0 or - response; N, total no. of sensilla tested.

cf. Table 4 for descriptions of SRI-C6, 2200-10, and 2504-5.

Repellents. Chemical substances classed as insect repellents appear to affect the neural activity of at least five of the six recognized antennal sensilla types. However, they do not evoke anything resembling a common response pattern among the various types of sensory neurons (Tables 4, 5, and 6). Sensory neurons of the short pointed sensory hairs of A. aegypti respond strongly to SRI-C6 and 612, and weakly to three other compounds. In contrast, the sensory neurons of the short blunt sensilla are strongly excited by 612, SRI-C6, compound 2504-5, citronellol, and naphthalene. Indalone and dimethylphthalate evoked weak responses in a few cases and failed to elicit a direct response in the remaining cases. SRI-C6, 612, and citronellol evoked relatively strong excitation responses in the sensory neurons of the short blunt sensilla in all 4 species. In A. aegypti, A. stephensi, and C. tarsalis, naphthalene excited the neurons in the short blunt sensilla. The neurons in the long blunt sensory hairs (A2-I) were unresponsive to the insect repellents in this study, even though previous work had indicated that these neurons exhibited strong excitation to deet and 612 (Davis and Rebert, 1972). We cannot explain this apparent discrepancy in the response of this group of sensory neurons now. Thus, in the chemosensory neurons associated with the three types of antennal sensory hairs (sometimes called sensilla trichodea or A-2 type sensilla), there is no apparent pattern of neural activity associated with "insect repellents."

In the two types of LA-sensitive neurons, most repellent compounds elicit only weak responses directly from the sensitive neurons. As Davis and Sokolove (1976) showed, the insect repellents deet and 612 can interfere with the detection of LA by the LA-excited neurons.

To study the effect in more detail, we presented LA alone and together with different intensities of deet and 612, and plotted the response to LA, and to LA plus repellent against the stimulus intensity (Fig. 3). Both deet and 612 shifted the response curves to the right, i.e., higher intensities of LA were necessary to elicit a given level of response. Although the difference was not great, deet appeared to

Table 6. Responses of A2-type sensilla trichodea of four species of mosquitoes to repellent compounds. (all stimuli presented neat via syringe)

Species	Stimuli	Sensilla Responses: Short Pointed Resp.	Short Pointed No./N	Long Blunt (A2-I) Resp.	Long Blunt (A2-I) No./N	Short Blunt (A2-II) Resp.	Short Blunt (A2-II) No./N
Aedes aegypti	Deet	0	13/23	0	27/30	0	5/8
	Indalone	0	10/13	0	18/18	+	4/6
	612	++	12/16	0	18/23	++	4/7
	SRI-C 6	++	9/19	0	22/23	++	10/10
	2504-5	0	5/6	0	9/12	++	3/5
	Citronellol	+	7/11	0	22/22	++	5/7
	Geraniol	+	7/13	0	14/14	0	3/5
	Napthalene	0	8/8	0	9/10	++	4/5
	Juglone	+	4/5	0	5/7	0	2/2
	Vanillin	0	4/4	0	1/1	0	1/1
	Indole	0	1/1				
	Dimethyl phthalate	0	2/2				
A. tinseriatus	Deet	-	3/7	0	2/3	+/-	2/3
	Indalone	+	4/7	0	2/2	0	2/3
	612	0	3/5	0	2/3	++	2/3
	SRI-C 6	0	6/7	0	3/4	++	5/6
	2504-5			+	1/2	0	2/2
	Citronellol	0	2/3	+	2/4	++	2/2
	Geraniol	0	3/4	+	2/4	0	2/2
	Napthalene	0	1/1	0	1/1	0	3/3
	Juglone	0	1/1	+	1/1	0	1/1
	Vanillin	+	1/2	0	1/1		
	Indole	+	1/2				
	Dimethyl phthalate	++	1/2				
Anopheles stephensi	Deet	0	3/4	+/-	2/3	0	4/4
	Indalone	0	4/4	0	3/3	0	3/4
	612	0	4/5	0	3/3	+	3/5
	SRI-C 6	0	6/6	0	2/3	++	5/5
	2504-5	-	2/4	0	2/3	0	2/2
	Citronellol	+/-	2/4	+	2/3	+	1/2
	Geraniol	+	2/4	+	2/2	0	2/2
	Napthalene	+	1/2	0	1/1	++	2/3
Culex tarsalis	Deet	0	4/4	0	2/2	0	5/7
	Indalone	0	3/4	0	1/1	0	4/5
	612	0	4/4	+	1/2	++	6/8
	SRI-C 6	0	3/5	0	2/3	++	12/12
	2504-5	0	3/3	0	1/1	0	4/5
	Citronellol	0	5/6	0	2/3	++	9/11
	Geraniol	0	5/5	0	2/2	++	5/10
	Napthalene	0	3/4	0	1/1	+	5/6
	Juglone	0	2/2			0	2/3

cause a slightly greater shift than did 612. This shifting of the response curves was dose-dependent and suggests that the repellents in some way inhibit the response of the sensory neurons to LA.

To assess what type of inhibitory processes might be involved and to determine some of the kinetic properties of the LA-excited neurons, we replotted the data using a modified Lineweaver-Burke double-reciprocal plot of response as a function of stimulus intensity (Fig. 4). The resulting curves indicated that deet, at the intensities used (2.1 and 5.3 × 10^{-7} moles/min), acted as a competitive inhibitor of LA in the LA-excited neuron. At 5.3 × 10^{-7} moles/min, the repellent 612 also inhibited the action of LA in a competitive manner. At the lower stimulus intensity of 2.1 × 10^{-7} moles/min, 612 appeared to be either noncompetitive or intermediate between competitive and noncompetitive in its interaction with LA. However, more recent data suggest that 612 at the lower intensity may competitively inhibit the LA-excited response. This issue will be resolved only by replicating these studies at slightly different 612 intensities. In addition, for the LA-excited neuron, the double-reciprocal plots revealed a range of K_D values between 3 × 10^{-5} and 4 × 10^{-4} for LA alone. K_D values in this range are typical for enzymatic processes.

This confusing picture led us to re-evaluate how and where insect repellents act. The original assumptions were (1) that all insect repellents exert a similar influence on any given class of sensory neurons sensitive to them, and (2) that the behavioral response of the insect was also similar. We now feel that both these assumptions are incorrect. We have demonstrated that the repellents do not act as a class of compounds generating a similar response pattern in sensitive neurons. Quite the contrary, different repellent substances act in different ways on different groups of sensory neurons. Deet and 612 block activity in a host-sensitive neuron (LA-excited neuron); SRI-C6 and 612 mimic the response of oviposition attractants on the neurons sensitive to that class of compounds; while in a third sensitive-type (A2-I) they are excitatory (assuming that the results of Davis and Pebert are correct). Thus, one "repellent" may block a normally attractive response, another

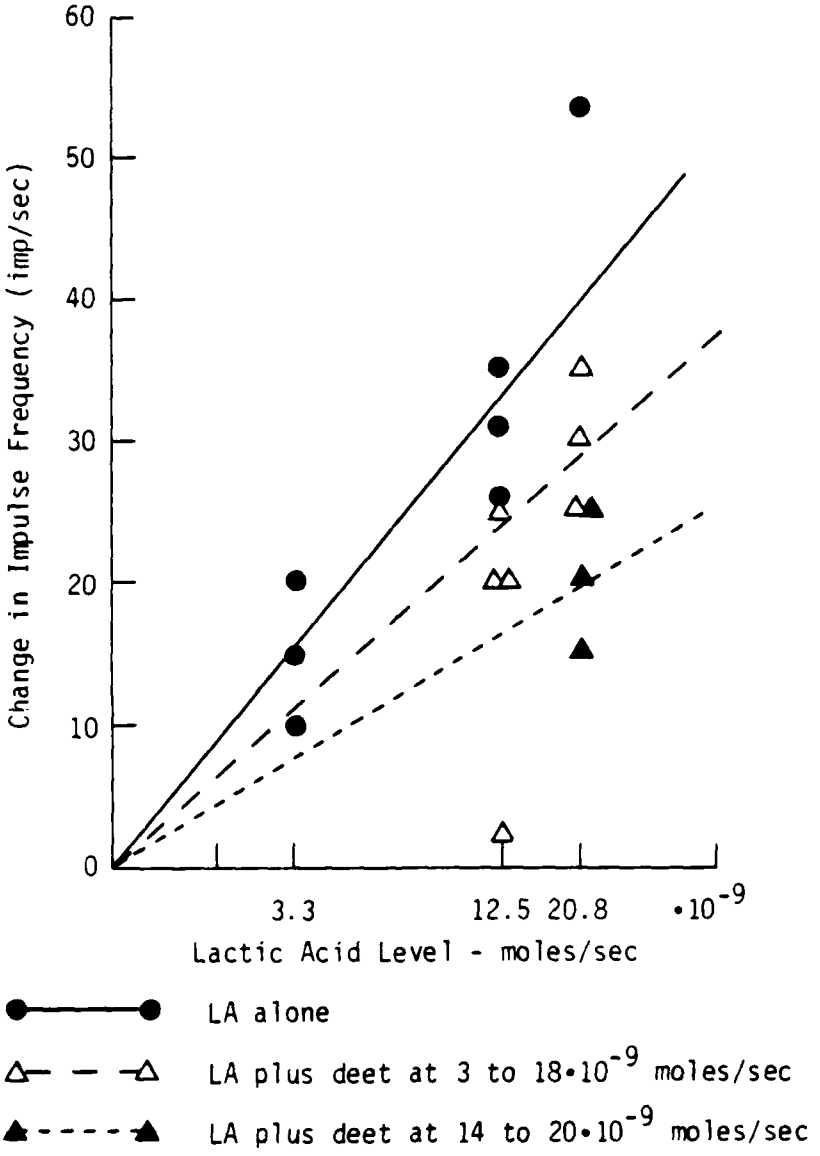
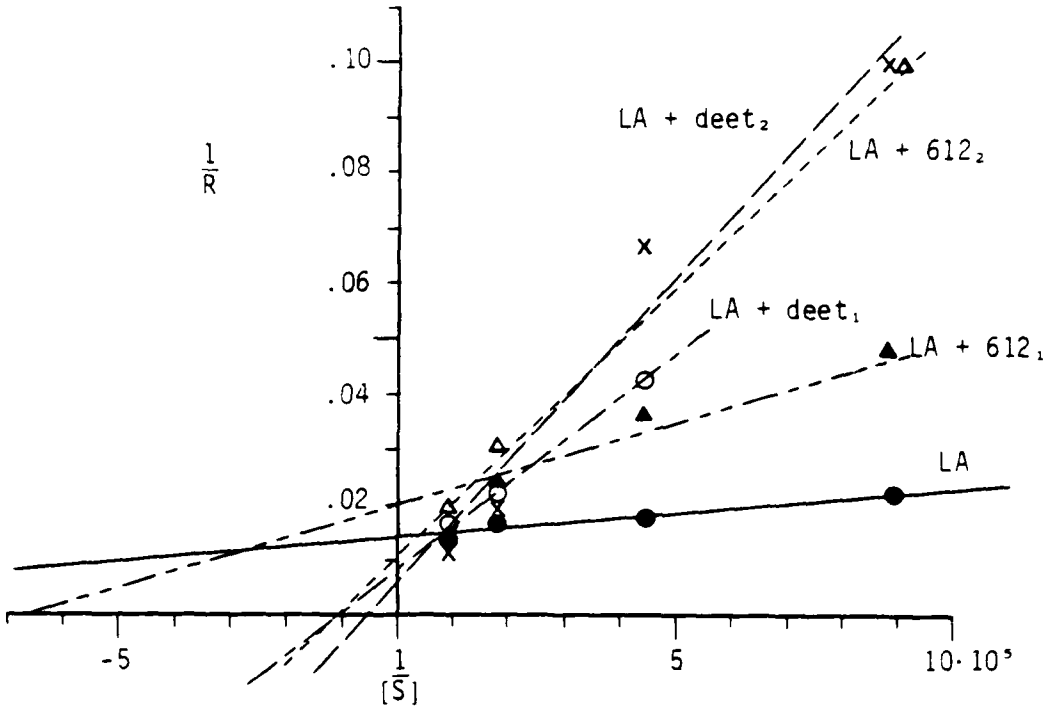


FIGURE 3 EFFECT OF THE INSECT REPELLENT, DEET, ON THE RESPONSE OF THE LACTIC ACID-SENSITIVE NEURONS OF THE ANTENNAL GROOVED-PEG SENSILLA OF Aedes aegypti



LEGEND:

Stimulus:	LA alone	LA + deet$_1$ at $2.1 \cdot 10^{-7}$ moles/min	LA + deet$_2$ at $5.3 \cdot 10^{-7}$ moles/min	LA + 612$_1$ at $2.1 \cdot 10^{-7}$ moles/min	LA + 612$_2$ at $5.3 \cdot 10^{-7}$ moles/min
K_D :	$7.1 \cdot 10^{-7}$	$9.1 \cdot 10^{-6}$	$1.7 \cdot 10^{-5}$	$1.5 \cdot 10^{-6}$	$8.3 \cdot 10^{-6}$
Rmax :	71	111	143	50	83
Correlation coefficient:	0.938	0.998	0.979	0.981	0.999
Type of Inhibition :	--	Competitive	Competitive	Intermediate: Noncompetitive-Competitive	Competitive

R = Response defined as the change in spike frequency in impulses/sec.

[S] = Stimulus intensity in moles/min.

All curves fitted to data using linear regression technique.

FIGURE 4 DOUBLE-RECIPROCAL PLOT OF THE RESPONSE OF A LA-EXCITED NEURON TO LA ALONE AND TOGETHER WITH DEET OR 612

"repellent" may cause an inappropriate behavior--e.g., oviposition--to be selected, and a third "repellent" may trigger a response in a neuron responsible for detecting "noxious" substances. A fourth mode of action is the 'auto-repellency' induced by a substance that is attractive at low concentrations, but repellent at higher concentrations. Para-cresol is one such compound for gravid female A. triseriatus (Bentley 1979).

To further complicate matters, the exact behavioral response to these substances is not clear. Compounds are classified as insect repellents on an operational basis, that is, if they prevent a female mosquito from biting a host. If our electrophysiologic data are biologically relevant to the modes of action of some of the "repellent" substances tested, then we would expect that different repellents might evoke different behavioral responses for the female mosquitoes. This would explain why chemists have failed to find similarities in their physical properties and common chemical structures that they could relate to repellent efficacy. Repellents apparently are not a class of compounds with a common critical set of properties, but are various compounds, with diverse properties and sites and modes of action, that evoke different behavioral reactions.

The notion of insect repellents clearly needs to be re-examined, the action of the substances on the behavior of the mosquito must be better defined, and more detailed quantitative studies of the action of "repellent" compounds on the peripheral sensory organs should be conducted. Only then will we understand how and where insect "repellents" act and how to enhance their use to protect ourselves against the disease-transmitting ability of the mosquito.

Objective 2

The sensitivity of the LA-excited neuron to LA, a normally attractive host compound, was markedly depressed after a blood meal. This alteration of receptor sensitivity followed a time course nearly identical to that observed for the inhibition of host-seeking behavior. After oviposition, the sensitivity of the LA-excited neuron returned

to that observed before the blood meal. Furthermore, the sensitivity of the antennal chemosensory neurons sensitive to the reported oviposition site attractant methylbutyrate (MB) increased at about the time that the oöcytes normally reach maturity and oviposition behavior is initiated. Thus, we demonstrated that changes in activity in the peripheral sensory system could account for the sequence of behaviors normally exhibited by a female mosquito after she has fed on blood.

This is the first direct evidence for humoral modulation of peripheral sensory organs resulting in the control of an organism's behavior.

A detailed account of these experiments is presented in the appendix manuscript entitled "Regulation of Sensitivity in the Peripheral Chemoreceptor Systems for Host-seeking and Oviposition Behavior by a Hemolymph-borne Factor in the Mosquito, Aedes aegypti," which was submitted to the Journal of Insect Physiology.

Other Work

Although support for this project was discontinued and we therefore did not have time to accomplish Objective 3 of the originally proposed work (scheduled for Years 2 and 3), we developed expertise in obtaining reliable measures of nerve-discharge activity in contact chemoreceptor organs.

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PUBLICATIONS/PRESENTATIONS
Resulting from this Project

Takahashi, F. T., Jr., and E. E. Davis. Effect of blood feeding on the responses of the antennal chemoreceptors and behavior in Aedes aegypti. Presented at the Ann. Mtg. Entomol. Soc. Amer., Denver, Colorado, 26-29 November 1979.

_____. Humoral alteration of chemoreceptor sensitivity in Aedes aegypti following a blood meal. Int. Conf. on Regul. of Insect. Develop. Behav., Karpacz, Poland, 23-28 June 1980. (Accepted but not presented.)

Davis, E. E. and F. T. Takahashi, Jr. Humoral alteration of chemoreceptor sensitivity in the mosquito. Int. Symp. Olf. Taste VII/ European Chemoreceptor Res. Org. IV Joint Mtg., Noordwijkerhout, Holland, 21-25 July 1980. (Appended.)

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Davis, E. E. Regulation of sensitivity in the peripheral chemoreceptor systems for host-seeking and oviposition behaviors by a hemolymph-borne factor in the mosquito, Aedes aegypti. (Submitted to J. Insect Physiol.) (MS Appended)

_____. Structure-activity relationship of lactic acid-excited afferent neurons in the antennal grooved-peg sensilla of the mosquito, Aedes aegypti. (Submitted to J. comp. Physiol.) (MS Appended)

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_____. Sensory physiology and mechanisms of repellency. In A Symposium on Attractancy and Repellency of Mosquitoes. Amer. Mosq. Control Assoc. Ann. Mtg., San Antonio, Texas, 15-18 March 1981.

Appendix 1: Final Report--
Neurophysiological Study of
Vector Responses to Repellents

E. E. Davis

Humoral alteration of chemoreceptor sensitivity in the mosquito

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ABSTRACT

Electrophysiologically, we have measured changes in the sensitivities of single antennal neurons from female mosquitoes that coincide with behavioral changes reported to occur within 24 h after a blood meal. The lactic acid (LA) excited neurons become less sensitive to LA--a host attractive substance-within 24 h post blood meal. At the same time, neurons sensitive to oviposition site attractant become more sensitive to chemical stimuli reported to attract gravid females to oviposit. Hemolymph-transfusion studies suggest that the hemolymph-borne factors responsible for the reported behavioral changes are also responsible for the observed alterations in receptor sensitivities.

INTRODUCTION

In female mosquitoes, the taking of a blood meal results in the inhibition of host seeking and biting behaviors.[1] This host-refractory behavior is reported to be due to hormonal changes that occur within a few hours after a blood meal.[2] Host-seeking and other behaviors are complex integrated activities resulting from processes in the mosquito's CNS and are mediated--in part--by input from the chemosensory neurons on the antennae of the mosquito. Any change in the responsiveness of these peripheral sensory organs might affect the behavior of the organism. We present here electrophysiological evidence for a potential mechanism underlying changes in the sensitivities of certain antennal chemosensory neurons following a blood meal that could help account for the altered host-seeking behavior.

METHODS

Adult male and female Aedes aegypti were caged together and maintained on 5% sucrose for 7 days; then the females were allowed water only for 24 h before they were allowed to feed to repletion on a guinea pig.

Standard electrophysiological techniques were used to detect, amplify, and record the extracellular action potentials (spikes) of the chemosensory neurons.[3]

Airborne chemical stimuli were generated in one of two ways. One method

was to apply neat compounds to small pieces of filter paper placed in individual 10-ml syringes. The vapors of the test compounds in the syringes were expelled manually over the mosquito. The other method of stimulus generation was to pass air through a solution of the test substance in a flask. The stimulus intensity was controlled by individual flow meters and metering valves. The mosquito was stimulated by activating a solenoid valve that routed the appropriate odor stream over the mosquito.

To transfuse hemolymph, a micropipette mounted on a micromanipulator was connected by polyethylene tubing to a ml remote syringe. The .1 ml of hemolymph injected into non-blood-fed female mosquitoes was collected either from 6-8 non-blood-fed females (control) or 6-8 blood-fed females 24, 48, or 72 h post blood meal (PBM). After 2 h, electrophysiological recordings were begun.

We recorded the responsiveness of the lactic acid (LA)-excited neurons in the antennal grooved-peg sensilla and the oviposition site attractant-sensitive neurons in the short, pointed sensory hairs. The physiological characteristics of these sensory neurons were described in detail in the literature. [1] In this study, we were concerned with the potential changes in the firing characteristics of the sensory elements that might occur after a female mosquito took a blood meal. The specificity of the respective sensory neurons was examined by presenting a broad range of chemical substances to the mosquito. Changes in receptor sensitivity were determined by comparing the stimulus intensity vs neural response curves for individual neurons from control and experimental hemolymph-injected female mosquitoes.

RESULTS AND DISCUSSION

Neural responses obtained from the LA-excited neurons did not show any change in their specificity for chemical substances reported to evoke changes in their firing frequency. We did observe changes in the sensitivity of these neurons to LA following blood feeding. Fig. 1 shows the stimulus intensity vs response functions for 16 LA-excited neurons to LA. As early as 24 h PBM and lasting through 96 h PBM, the sensitivity of the LA-excited neurons to LA was reduced. Because none of these females were allowed to oviposit, no return to the sensitivity levels of non-blood-fed mosquitoes was expected; none was observed up to 96 h PBM. In females allowed to oviposit, the sensitivity to LA showed signs of recovery to the levels in non-blood-fed females.

Similar measurements from oviposition-attractant-sensitive (OAS) neurons showed no change in the specificity for the chemical stimuli known to evoke

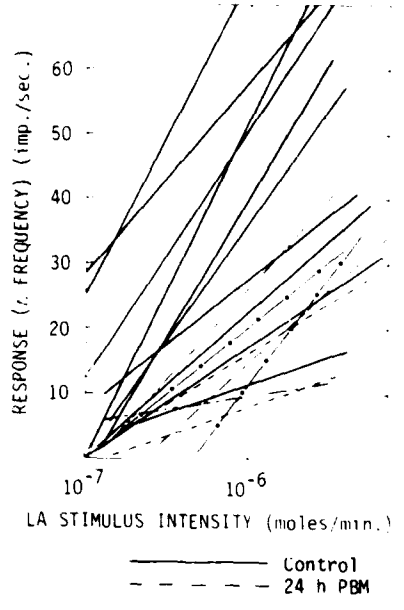

FIGURE 1 RESPONSE OF THE LA-EXCITED GROOVE PEG SENSILLA TO LA IN PRE-BLOOD MEAL (CONTROL) AND 24, 48, AND 72 HOUR POST-BLOOD MEAL (PBM) Ae. aegypti FEMALES.

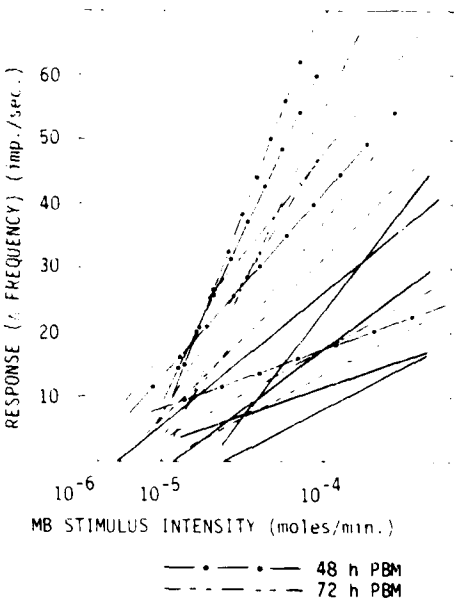

FIGURE 2 RESPONSE OF THE SHORT POINTED SENSILLA TO METHYL BUTYRATE (MB) IN PRE-BLOOD MEAL (CONTROL) AND 24, 48, AND 72 HOUR POST-BLOOD MEAL (PBM) Ae. aegypti FEMALES.

responses from them. A change in the sensitivity of these neurons was observed; they exhibited an increased sensitivity to reported chemical oviposition attractants for gravid female A. aegypti.[5] Fig. 2 shows the response functions of 17 OAS neurons to the oviposition attractant methyl butyrate (MB). The response functions clearly show that the OAS neurons of blood-fed females are more sensitive to MB than are their counterparts on non-blood-fed females.

The decreased sensitivity of the LA-excited neurons to the host-attractant LA coincides in time with the reported inhibition of host-seeking behavior in blood-fed mosquitoes.[6] The increased sensitivity of the receptors for oviposition attractants also coincides with oocyte maturation and the onset of oviposition behavior.[7]

Transfusion of hemolymph from blood-fed females into non-blood-fed mosquitoes resulted in changes in receptor sensitivities similar to those described above. The LA-excited neurons of the non-blood-fed females injected with hemolymph from 48-h-PBM females are considerably less sensitive to LA than those injected with hemolymph of non-blood-fed females (Table 1)

Table I: Comparison of the effects of a hemolymph-borne factor on responsiveness of LA-excited and oviposition attractant-sensitive [illegible]

	Condition of Donor Mosquitoes			
	Non-blood-fed	Blood-fed (Hours PBM)		
Receptor type	(Control)	24	48	72
LA-excited	44±8	49	19±3	29±4
	n = 14	n = 2	n = 5	n = 12
Oviposition attractant-sensitive	43±5	40±7	47	49±12
	n = 14	n = 3	n = 2	n = 5

*The data are the mean responses (± SEM) to single intensities of LA and MB selected from their respective stimulus intensity-response curves. The intensity levels were those at which the respective samples of LA- and MB-sensitive neurons were most active (LA: 13 x 10^{-7} moles/min. MB: 22 x 10^{-6} moles/min).

A slight increased sensitivity to methyl butyrate is evident in females receiving hemolymph of 72-h-PBM females. These data indicate that the same hemolymph-borne substance that affects the behavior patterns of the mosquito also acts on the receptors mediating those behaviors. Thus, the peripheral sensory neurons of the mosquito could play an important role in modifying its behavior. The decreased sensitivity to chemical signals associated with a behavior that is no longer appropriate--i.e., host-seeking following a blood meal--and the increased sensitivity to signals associated with a behavior that is now appropriate--i.e., oviposition--may be important factors in mediating the observed behavioral changes. Although other investigators have suggested that neurosecretory substances may alter chemoreceptor activities in other insect systems,[7,8] the data presented here are the first direct evidence for such an occurrence.

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Appendix 2: Final Report--
Neurophysiological Study of
Vector Responses to Repellents

E. E. Davis

REGULATION OF SENSITIVITY IN THE PERIPHERAL CHEMORECEPTOR SYSTEMS FOR HOST-SEEKING AND OVIPOSITION BEHAVIORS BY A HEMOLYMPH-BORNE FACTOR IN *AEDES AEGYPTI*

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Running Head: Humoral Control of Receptors

Key Words: *Aedes aegypti*, mosquito, chemoreceptors, host-seeking behavior, oviposition behavior, hormones, receptor regulation

ABSTRACT

The taking of a blood meal and subsequent development of eggs by a female mosquito is reported to suppress host-seeking behavior. This change in behavior may be partly mediated by changes in certain chemosensitive antennal afferent neurons that influence the behavior of female mosquitoes. Electrophysiological activity of the lactic acid (LA)-excited neurons to LA--a normal host attractant substance--is depressed following a blood meal, whereas that of the methyl butyrate-sensitive neurons is increased. This reduction in LA sensitivity is coincident with the reported inhibition of host-seeking behavior. The reduction in LA sensitivity is reversible; this sensitivity returns to the pre-blood-fed level following oviposition. Like the inhibition of host-seeking behavior, the reduced LA sensitivity is due to a transfusable, hemolymph-borne factor. That the effect of blood feeding on the sensitivity of the antennal afferent neurons to LA is confined to the LA-excited neuron is evidenced by the lack of effect of the blood meal on the LA-inhibited neuron. A role for the peripheral sensory system in the control of behavior in female mosquitoes is discussed.

INTRODUCTION

Complex integrated behavior is primarily the result of processes in the central nervous system of an organism. However, the peripheral sensory organs play a decisive role in that they provide information on which the CNS can operate. Therefore, any change in the responsiveness of the peripheral sensory organs would be expected to affect the behavior of the organism.

Recent reviews of the cellular mechanisms for modulating behavior have provided many examples of how hormones act on CNS processes to modify the behavior of both vertebrate and invertebrate organisms (Truman and Riddiford, 1977; Truman, 1978; Kandel et al., 1979; Bloom, 1980). No cases were mentioned in which a hormone modulated the behavior of an animal by acting on a peripheral receptor system. The literature on insect sensory systems contains only two examples that even suggest such a peripheral effect. In one study, Bernays and Chapman (1972) reported that a substance from the corpora cardiaca of recently fed Locusta migratoria acts directly on the sensilla of the maxillary palps, presumably causing the closure of terminal pores of the sensilla, thereby indirectly reducing the sensitivity of the gustatory receptors following feeding. In the other study, Palaniswamy et al. (1979) reported that Altosid$_{TM}$, a juvenile hormone mimic, applied topically to male spruce budworm moths, reduced the amplitude of the electroantennogram

(EAG) response to its sex pheromone. They suggested that the reduced EAG was the result of a decrease in the sensitivity of the pheromone receptors following application of the hormone-like substance.

Several investigators have noted that female mosquitoes that have fed to repletion will not engage in further host-seeking behavior (Edman et al., 1975; Klowden and Lea, 1978). One component of this host-refractory behavior occurs immediately and is the result of abdominal distention, independent of the substance ingested (Klowden and Lea, 1979a). A second component of host-seeking inhibition, also reported by Klowden and Lea (1979b), accompanies oöcyte development, appearing 24 to 30 hr post-blood meal (PBM) and reaching maximal effect between 36 and 72 hr PBM. Normal host-seeking activity is restored within 24 hr following oviposition. Their experiments with female Aedes aegypti mosquitoes involved both surgical manipulation of hormone-secreting tissues and hemolymph transfusions and clearly demonstrated that a hemolymph-borne substance present during oöcyte development was responsible for inhibiting the response toward a host. There is no direct evidence that oviposition behavior is initiated by the same hemolymph-borne factors that inhibit host-seeking behavior. However, as oviposition is the behavioral sequel of a blood meal, it seems logical that certain aspects of oviposition-related behavior are linked to the series of hormonal changes known to occur during oöcyte development.

In an attempt to determine whether the hemolymph-borne factors appearing after a blood meal act on the peripheral receptor organs or at more centrally located sites, I examined the responsiveness of two antennal chemoreceptor systems whose activity would most likely be affected

by the female mosquito having successfully fed on a host: the host attractant (lactic-acid)-sensitive neurons of the grooved peg sensilla and the oviposition site attractant-sensitive neurons of the short, pointed sensory hairs (A2). In this report, I present electrophysiological evidence for changes in the responsiveness of these two receptor systems following a blood meal that could be significant mediating factors for the observed inhibition of host-seeking behavior and subsequent oviposition site-seeking behavior. Furthermore, I will show that the modulation of chemoreceptor sensitivity is under the control of a hemolymph-borne factor(s) similar to that reported by Klowden and Lea (1979b) for host-seeking inhibition associated with oöcyte development.

METHODS

Adult male and female Aedes aegypti mosquitoes were caged together and maintained on 5% sucrose for 7 days, during which time nearly all of the females mated. The insectary in which the mosquitoes were reared was kept at 27°C and 70% R.H. Some females were allowed to feed to repletion on a guinea pig; others were kept on 5% sucrose. I examined the electrophysiological response characteristics of the lactic acid (LA)-sensitive and oviposition attractant-sensitive neurons of (a) females that had fed on sucrose only (nonblood-fed), (b) blood-fed females 24, 48, 72, and--in the case of the LA-excited neuron--96 hr PBM, and (c) blood-fed females 24-72 hr after they had been allowed to oviposit.

Standard electrophysiological techniques were used to detect, amplify, and record the extracellular action potentials (spikes) of the chemosensory neurons (Davis and Sokolove, 1976).

Airborne chemical stimuli were generated in one of two ways. One method was to apply neat compounds to small pieces of filter paper placed in individual 10-ml syringes. The vapors of the test compounds in the syringes were expelled manually over the mosquito. This method is used to test a large number of substances in a short period of time. The second method is used to present chemical stimuli in a graded intensity series at or near physiological levels. By this method, air is passed through a flask containing the test substance. The saturation of the odor air stream depends on the vapor pressure of the test substance at the temperature used. The stimulus intensity, expressed as the flux of stimulus over the antenna in moles/sec, was controlled by individual flow meters and metering valves. The mosquito was stimulated by activating a solenoid valve that routed the appropriate odor stream over the mosquito.

To transfuse hemolymph, a micropipette mounted on a micromanipulator was connected by polyethylene tubing to a micrometer syringe. The 0.5 µl of hemolymph injected into nonblood-fed female mosquitoes was collected either from 6-8 nonblood-fed females (control) or from 6-8 blood-fed females 24, 48, or 72 hr PBM. The electrophysiological recordings were begun 2 hr after injection of hemolymph.

I recorded the responsiveness of the LA-sensitive neurons in the antennal grooved-peg sensilla and the oviposition site attractant-sensitive neurons in the short, pointed sensory hairs. The physiological characteristics of these sensory neurons have been described in detail in the literature (Davis and Sokolove, 1976; Davis, 1977). In this study, I was concerned with the potential changes in the firing characteristics of the sensory

elements after a female mosquito took a blood meal. The specificity of the respective sensory neurons was examined by presenting a broad range of chemical substances to the mosquito. Changes in receptor sensitivity were determined by comparing the stimulus intensity-neural response curves for individual neurons from control and experimental blood-fed and hemolymph-injected female mosquitoes.

RESULTS

Effects of Blood Feeding on the LA-Sensitive Afferent Neurons

A comparison of the stimulus intensity-response relationships of the LA-sensitive neurons in the antennae of adult female A. aegypti mosquitoes in nonblood-fed, blood-fed, and oviposited conditions revealed changes in the response patterns of these neurons associated with these different conditions. Figure 1 is a plot of the electrophysiological response--i.e., the change in neural discharge frequency--vs. the intensity of LA in the stimulus air stream for the LA-excited neurons on (a) females fed sucrose only, (b) blood-fed females 24,48, 72, and 96 hr PBM, and (c) blood-fed females following oviposition. As shown in Figures 1A and B, the taking of a blood meal resulted in reductions in the slope and the response magnitude of the LA-excited neurons to the host attractant, LA, over the range tested. This flattening of the response curves is indicative of a decrease in the sensitivity of the LA-excited neurons to LA. Table 1 presents a comparison of the response values for all experimental conditions at a single stimulus intensity. The stimulus intensity selected for this comparison ($10 \cdot 10^{-3}$ moles/sec) was one at which most of the neurons responding were in the region of greatest slope change of their respective

(Fig. 1)

(Table 1)

stimulus intensity-response curves. From these data, we can see that the decrease in LA sensitivity is progressive, appearing as early as 24 hr PBM, reaching its maximum (i.e., minimum sensitivity) at 48 hr PBM, and continuing for at least 96 hr PBM. If gravid females, which would normally exhibit this decrease in LA sensitivity, were allowed to oviposit, the observed electrophysiological response of the LA-excited neurons approached the level of sensitivity observed in the LA-excited neurons in the nonblood-fed females 24 to 72 hr after oviposition (Figure 1C and Table 1). The results of Student's t-test (Table 1) indicate that the decrease in LA sensitivity of the LA-excited neurons in females at 48 hr PBM compared to that of nonblood-fed females is significant at $p < 0.001$, whereas the recovery of sensitivity, compared to the mean PBM level ($\bar{x}_{PBM}$), is significant at $p < 0.01$.

To determine whether these effects were common to both types of LA-sensitive neurons or were confined to the LA-excited neuron, we examined the neural discharge patterns of LA-inhibited neurons from female A. aegypti, using the same experimental conditions--and in some cases, the same mosquito--as for the LA-excited neurons. There was no measurable change in the sensitivity of these afferents to LA in females allowed to blood-feed. Furthermore, a comparison across all experimental conditions and between LA-excited and LA-inhibited neurons indicated that the spontaneous activities of these cells were not altered following blood-feeding or oviposition.

Effects of Blood Feeding on the Oviposition Attractant-Sensitive Afferent Neurons

The response characteristics of the neurons associated with the short, pointed sensory hairs (A2), when presented with the reported oviposition attractant methyl butyrate (MB), were also different before and after the female mosquito had taken a blood meal (Figures 2A and 2B). (Fig. 2) The stimulus intensity-response functions obtained from the MB-sensitive neurons following a blood meal were faster rising (steeper curve) and reach a higher maximum compared with stimulus intensity-response curves from nonblood-fed females, indicating an increase in the sensitivity of these neurons to MB after a blood meal.

To better demonstrate the differences in the response patterns of the populations of MB-sensitive neurons sampled from female mosquitoes either nonblood-fed or 48 hr PBM, it was necessary to plot the mean responses + s.d. for the two conditions together and examine the envelop of their standard deviations (Figure 2C). Even though such sample means from chemosensory neurons of mosquitoes do not indicate the response characteristics of individual neurons, they do give some insight into the overall response patterns of the population of MB-sensitive neurons sampled. Plotting the data in this way, we noted that over the MB intensity range of 0.05 to $0.5 \cdot 10^{-6}$ moles/sec, the sensitivity of the MB-excited neurons is markedly greater 48 hr PBM than prior to a blood meal. However, as the MB intensity is increased from 0.5 to $5.0 \cdot 10^{-6}$ moles/sec, the differences in the response patterns of MB-sensitive neurons from nonblood-fed and 48 hr PBM females become negligible.

The increase in sensitivity of the MB-excited neurons was slower in developing than the decrease in sensitivity of the LA-excited neurons, appearing after 24 hr PBM and reaching a maximum about 48 hr PBM. By 72 hr PBM, the MB sensitivity was well below the maximum recorded at 48 hr PBM (Table 2). The sensitivities at 48 and 72 hr PBM were significantly higher than for nonblood-fed levels at $p < 0.001$ and 0.01, respectively. We did not observe any apparent return of the PBM sensitivity to nonblood-fed levels 24 to 72 hr following oviposition (Table 2).

(Table 2)

Effects of Hemolymph Transfusion

To determine whether a hemolymph-borne factor might be involved in the reduction of sensitivity of the LA-excited neurons to LA and the increase in sensitivity of the MB-sensitive neurons to MB, we transfused hemolymph from both blood-fed and nonblood-fed female mosquitoes into females that had been fed only sucrose. Two hours following the transfusion, we examined the stimulus intensity-response relationships of the LA-excited neurons in the recipient females. Figure 3 depicts the stimulus-response functions for the LA-excited neurons in those females receiving hemolymph from nonblood-fed females (A) and in those receiving hemolymph from blood-fed females 48 hr PBM (B). The comparison of these two sets of stimulus-response curves reveals a reduction in the sensitivity of the LA-excited neurons to LA in those females receiving hemolymph from females 48 hr after they had taken a blood meal; this reduction is strikingly similar to that observed directly in the LA-excited neurons of blood-fed females (Figure 1). The t-test did not show statistically significant differences between the effects of transfusing hemolymph from nonblood-fed and blood-fed female mosquitoes. The reason for this may be that

(Fig. 3)

the injection of hemolymph resulted in a larger number of LA-excited neurons having stimulus-response curves in the lower portion of their range of distribution (compare Figure 1A with Figure 3A). This resulted in compression of the data and a marked overlapping of the stimulus-response curves between the experimental and control conditions. Even so, it should be noted that for the LA-excited neurons, four of the five stimulus-response curves for females receiving hemolymph from blood-fed females (experimentals) are in the lower 15% of the range of stimulus-response curves for females receiving hemolymph from nonblood-fed females (controls). Therefore, I feel that even though the difference between the effects was not statistically significant, the data were biologically significant.

Unlike the LA-excited neurons, the sensitivity of the MB-excited neurons was not markedly changed by hemolymph transfusion from 48 hr-PBM donors. Thus, either the causal factor for altering the sensitivity of the MB receptor is not present in the hemolymph 48 hr PBM or the time required to achieve the change in sensitivity is longer (> 2 hr) in the case of the MB-sensitive neurons.

Effects of Blood Feeding on the Specificity of the LA-Sensitive and MB-Sensitive Neurons

A comparison of the specificity of the LA-excited and LA-inhibited neurons to a wide variety of chemical stimuli that were presented via syringe showed no difference between responses obtained from nonblood-fed and blood-fed female mosquitoes. A similar comparison of the specificity of the MB-sensitive neurons to a broad spectrum of chemical stimuli indicated no difference between neurons on blood-fed and nonblood-fed females.

The series of chemical stimuli used for these qualitative estimations of specificities were essentially the same as those reported by Davis and Sokolove (1976) for the LA-sensitive neurons and Davis (1977) for the oviposition-sensitive neurons.

DISCUSSION

Peripheral Control of Behavior

The coincidence of the effects of a blood meal on the host-seeking behavior of a mated female mosquito and on the LA-excited neurons that provide information for the location and tracking of a host suggests that the peripheral sensory system may exert a strong influence over the behavior exhibited by female mosquitoes. It is generally accepted that the peripheral sensory system provides information about an organism's environment on which the CNS can operate in the development of complex behavior patterns. However, the important point here is that under normal, physiological conditions, these input channels are modified in such a way as to affect the quantitative flow of information to the CNS, and the modification thereby becomes a significant factor in the control of the overt behavior of the organism. This is in contrast to the notion that the information-transmitting capacity of the peripheral afferent neurons remains unchanged and that control mechansism in the CNS determine what information will or will not be used (Kandel et al., 1979). Although the data presented here do not preclude any potential integrative activity in the CNS, they are, I think, indicative of a significant role for the peripheral sensory system in the selection of a behavior pattern as well as in the modulation of a behavior pattern already selected.

I propose the following hypothesis to account for the coincident sensory-behavioral changes associated with the taking of a blood meal and subsequent oögenesis. Receptors of the peripheral sensory system mediate host-locating behavior prior to the female having obtained a blood meal in the following way. Initially, the female mosquito engages in patrolling activity or waiting for a potential host. When the appropriate host-related stimuli are perceived, she switches from patrolling or waiting behavior to active host-locating and tracking behavior. If she loses the trail of the host-related stimuli, she will revert back to patrolling or waiting behavior. These switches in behavioral modes are mediated solely by quantitative changes in the sensory input--i.e., the relative presence or absence (the level of stimulus is either above or below behavioral threshold) of certain airborne host stimuli--provided the CNS by the peripheral sensory system. Now, if there is a change in the sensitivity of one type of receptor such that the amount of information transmitted to the CNS is below the behavioral threshold, then the behavioral mode normally mediated by that receptor system cannot be expressed even though the appropriate stimuli for that receptor may still be present. Conversely, if the sensitivity of the receptor system is enhanced, the behavior mediated by that receptor system becomes easier to elicit. Thus, a change in the intrinsic sensitivity of a single receptor system may favor one behavior over another. Carrying the argument one step further, if the activity of one receptor system is enhanced while a second is depressed, the potential for the peripheral sensory system to cause the

selection of one behavior over another becomes quite clear. In the mosquito, we must add an additional factor--i.e., synergism between two receptor systems that mediate the same behavior.

Acree et al. (1968) and Smith et al. (1970) found that LA and CO_2 act synergistically to enhance the behavioral attraction of unfed female mosquitoes. This synergism is the result of integrative processes in the CNS acting on the input from separate LA- and CO_2-sensitive afferent neurons (Davis and Sokolove, 1976). Given this synergistic interaction, if the input from one or the other afferent pathways is altered (in this case, the LA-excited neurons), the effect on the behavior will be much greater than that for a similar change in a single receptor system. Thus, what may appear to be a modest change in the activity of the LA-excited neuron has the potential to mediate a significant change in the host-seeking behavior of the female mosquito. Following a blood meal, the sensitivity of the LA-excited neuron could be lowered to such a level that, even at airborne LA levels normally signaling the presence of a potential host, the information provided to the CNS by the LA-excited neuron is below the threshold level for initiating host-seeking behavior.

The increase in the sensitivity to MB observed in the set of receptors responding to oviposition site attractants following the blood meal has a longer latency (48 hr PBM) than does the change in sensitivity of the LA-excited neuron. The change in MB sensitivity may switch the behavior of the female from a relatively inactive mode to an oviposition site-seeking mode when the oöcytes are normally reaching maturity and are becoming ready for deposition.

After oviposition has been completed, the sensitivity of the LA-excited neuron returns to preblood-fed levels, thereby increasing the capacity of the neurons to transmit sensory information for host-seeking behavior. If, at the same time, there were a decrease in the input for oviposition-related stimuli, which, unfortunately, we have not yet been able to show, then the reversal of these peripheral sensory inputs would allow host-seeking behavior to again be expressed while oviposition behavior is suppressed.

The results of these studies strongly suggest that changes in the activities of the discrete elements in the peripheral sensory system are sufficient to account for the selection and modulation of behavior in the mosquito and that it is not necessary to invoke complex integrative activity in the CNS to explain these phenomena.

Potential Mechanisms and Sites of Action

The change in the sensitivities of the LA-excited and MB-sensitive afferent neurons and the inhibition of host-seeking behavior following a blood meal are mediated by a hemolymph-borne factor, quite possibly the same factor. Klowden and Lea (1979b) have presented evidence suggesting that the ovaries may be the source of the substance. That the substance is ecdysterone (20-OH-ecdysone or β-ecdysone), as postulated by Beach (1979), is unlikely because if one transfuses hemolymph from fed to unfed females at the time of greatest effect, 48 hr PBM, the titer of ecdysterone is at a low level--similar to the level prior to the blood meal when the LA sensitivity is high and host-seeking behavior is observed. In Culex spp. females, because high levels of juvenile hormone induce biting

behavior (Mecla and Petralia, 1980), one could argue that low levels [illegible] hibit biting. However, because the substance causing host-seeking inhibition can be transferred in the small amount of hemolymph taken from [illegible] fed females that develop eggs (Clowden and Lea, 1979b), the sensory and behavioral changes are due to the presence of a hemolymph-borne substance rather than because its level is below some threshold value.

Within the grooved-peg sensillum, the action of the hemolymph-borne factor responsible for the sensory changes observed following a blood meal is confined to the LA-excited afferent neuron. Neither the LA-inhibited afferent nor the spontaneous activity of either type of LA-sensitive neuron showed any change in the level of activity or responsiveness under the conditions that caused the decrease in sensitivity of the LA-excited afferent neurons. In the short, pointed sensilla, we cannot detect any difference in the activities of the two MB-sensitive neurons present. From previous work we know that there are two sensory neurons in this sensillum (McIver, 1978) and that the two neurons respond in a similar manner to MB (Davis, 1977). Therefore, we conclude that the hemolymph-borne factor acts on both MB-sensitive neurons in the same way.

Several potential mechanisms could account for the changes in the sensitivities of the two peripheral receptor systems studied here. Wieczorek (1980) and Thurm and Wessel (1979) have suggested that an electrogenic K-pump in the sensillar sheath cells may act as an added driving potential necessary for the dendritic generator potentials to travel the "relatively long distances" between the area of the primary receptor processes and the spike-generating region. Such a driving potential would be expected

to exert its effect on all the cells within the sensillum. As a result, one would also expect to see a change in the sensitivity of the LA-inhibited neurons, but I did not observe it. Therefore, this potential mechanism is an unlikely candidate within the receptor systems described here. However, it is possible that through electrotonic coupling between the sheath cells and, for example, the LA-excited afferent neuron, either the cell membrane becomes hyperpolarized or the threshold for spike initiation is raised so that the spike-generating ability of that cell would be reduced. This would be reflected as an apparent decrease in the sensitivity of the LA-excited neuron for LA. However, the observation that stimuli unrelated to LA, such as NH_3 are equally able to cause the initiation of nerve spikes before and after blood-feeding refutes this hypothesis (Davis, unpublished observations).

Another series of potential mechanisms deals with the ability of the receptor sites on the LA-excited and MB-sensitive neurons to interact with their respective ligands through a change in the number of available receptor sites in the cell membrane. In several systems, most notably vertebrate gonadotropic hormones, the number of available receptor sites has been shown to change following exposure to hormone (Tata, 1977; Melnechuk, 1978; Kandel et al., 1979). The mechanisms by which the number of receptor sites may vary in these systems requires that the regulating hormone affect the rates at which new receptor proteins are synthesized and replace those receptor proteins undergoing normal or accelerated turnover. Half-times of 10 to 30 hr are associated with these processes. In the mosquito, we can obtain the change in sensitivity in the LA-excited neurons within 2 hr following transfusion of

hemolymph from blood-fed to nonblood-fed females. This observation argues against such changes in the rates of receptor synthesis and/or turnover as the mechanism for altered sensitivity of the LA-receptor system in A. aegypti.

Thus, from the foregoing, it appears that the hemolymph-borne factor inhibiting host-seeking behavior that is present following a blood meal must act rapidly--i.e., within 2 hr--and directly on the receptor regions of the LA-excited reurons. The mechanism(s) by which this factor affects receptor sensitivity must await further investigation.

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Table 1. A comparison of the mean responses of the LA-excited neuron to LA for nonblood-fed, blood-fed, and oviposited female A. aegypti at a single intensity level of LA ($10 \cdot 10^{-9}$ moles/sec). Response values are the mean change in spike frequency (impluses/sec) for each condition ± the standard deviation and N is the number of sensilla tested in each condition. Blood-fed females were tested 24, 48, 72, and 96 hr PBM. Σ_{PBM} is the mean of all PBM trials. The probability functions were calculated using the Student's t-test for independent means; values for blood-fed were compared with those for nonblood-fed (P_a) and values at 24-72 hr post-oviposition were compared with Σ_{PBM} (P_b).

Condition of Female:	Mean Response (± s.d.)	N	P_a	P_b
Nonblood-Fed	50 ± 15	11	-	-
Blood-Fed (hours PBM)				
24	21 ± 7	3	.01	-
48	16 ± 9	5	.001	-
72	18 ± 13	3	.01	-
96	21 ± 5	2	.05	-
Σ_{PBM}	19 ± 8	13	.001	-
Post-Oviposition	35 ± 10	6	-	.01

Table 2. A comparison of the mean responses of the oviposition attractant-sensitive neurons to MB for nonblood-fed, blood-fed, and oviposited female A. aegypti at a single intensity level of MB (6.0 to $7.4 \cdot 10^{-7}$ moles/sec). Response values are the mean change in spike activity (impulses/sec) for each condition ± the standard deviation and N is the number of sensilla tested in each condition. Blood-fed females were tested 24, 48, and 72 hr PBM. Σ_{PBM} is the mean of all trials at 48 and 72 hr PBM. The probability functions were calculated using Student's t-test for independent means; values for nonblood-fed were compared with values for all conditions (P_a) and values at 24-72 hr post-oviposition were compared with Σ_{PBM} (P_b).

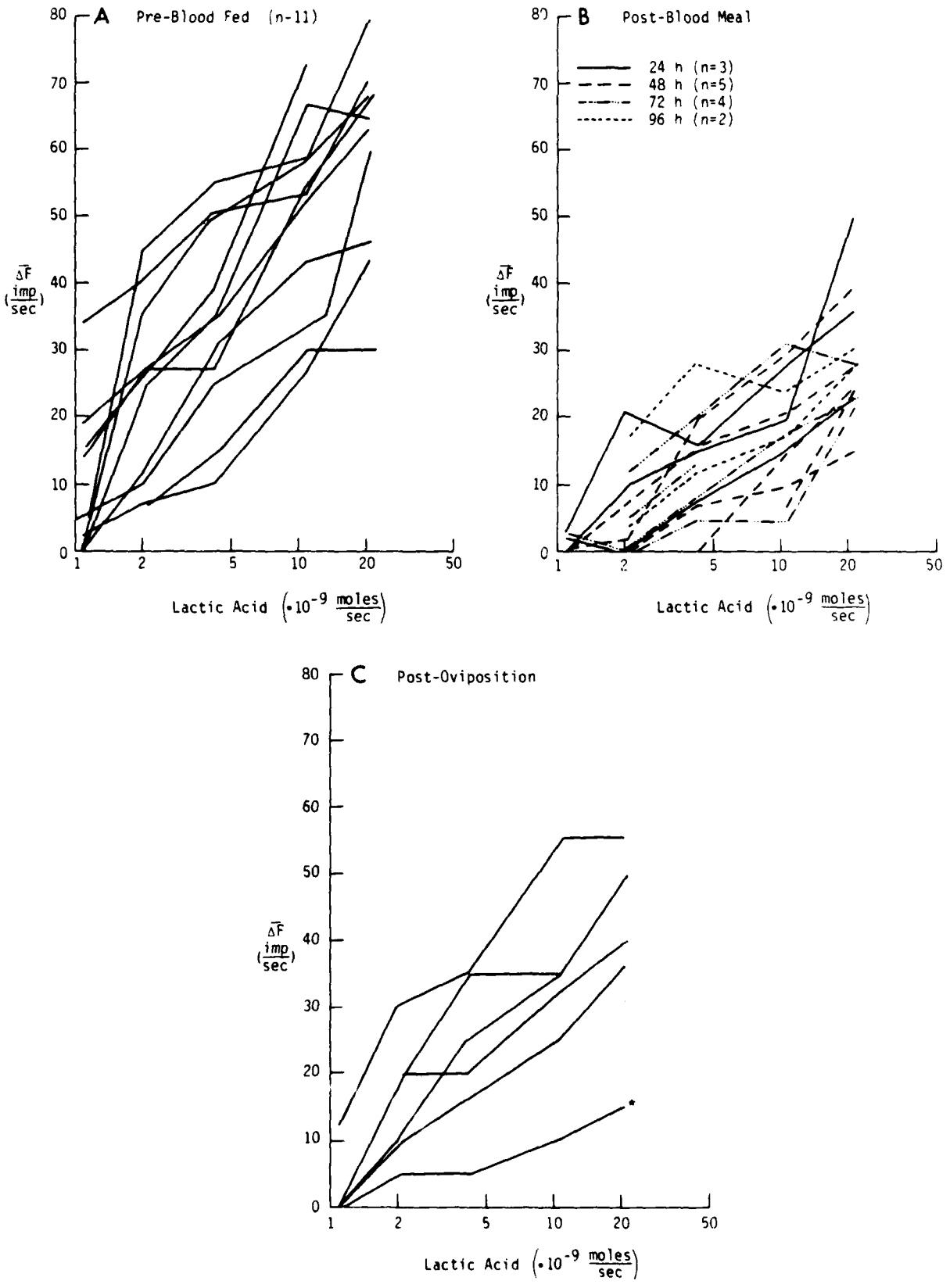
Condition of Female	Mean Response (± s.d.)	N	P_a	P_b
Nonblood-Fed	15 ± 9	18	-	-
Blood-Fed (hours PBM)				
24	16 ± 9	9	>.50	-
48	37 ± 17	7	.001	-
72	23 ± 7	5	.10	-
Σ_{PBM}	31 ± 15	12	.001	-
Post-Oviposition	29 ± 16	10	-	>.50

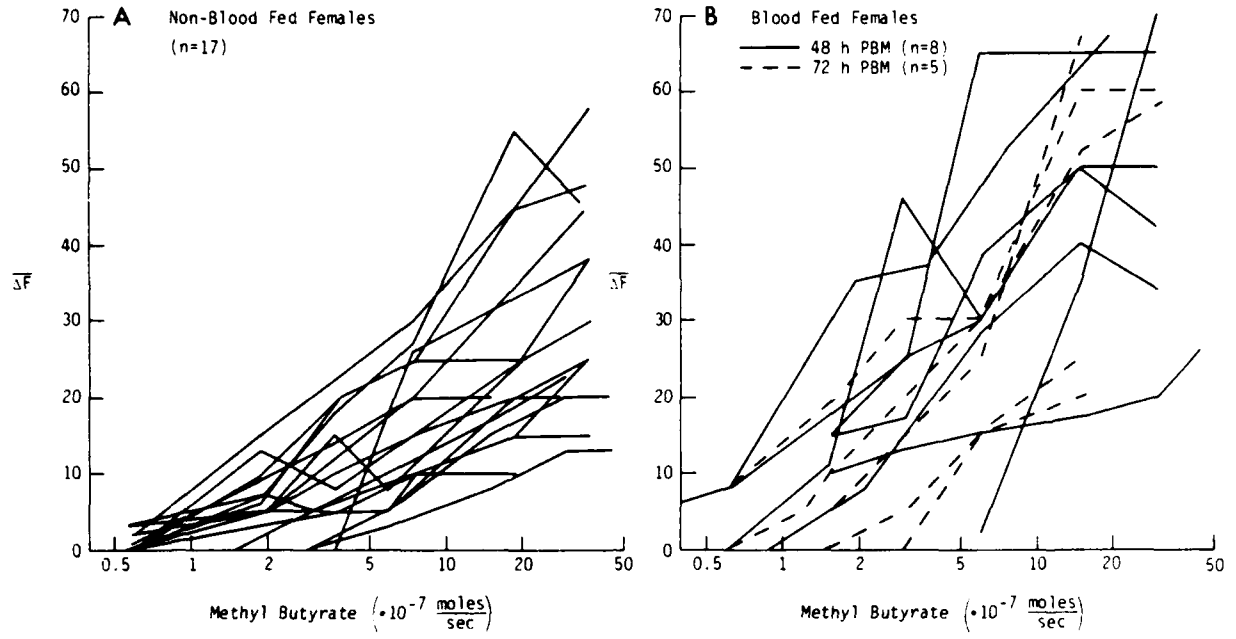
LEGENDS

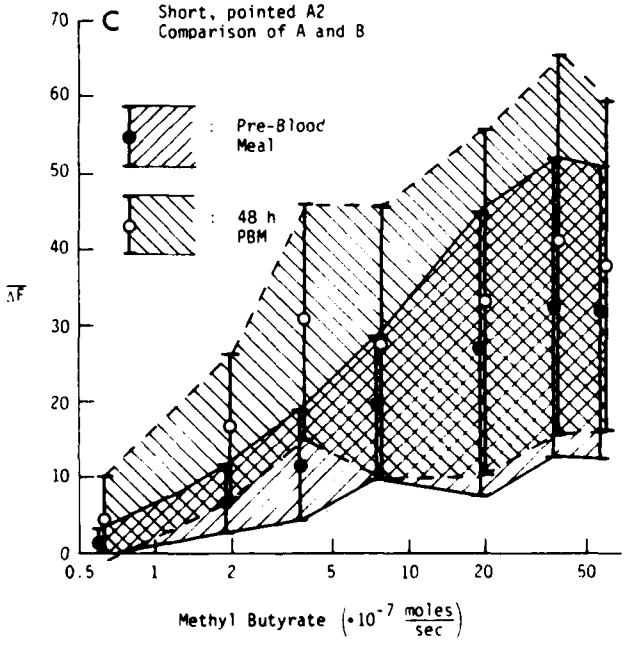
Fig. 1 Stimulus intensity vs. response functions of the LA-excited neurons to LA. The response functions were obtained from LA-excited neurons on (A) nonblood-fed females and (B) blood-fed females 24, 48, 72, and 96 hr PBM, and (C) females 24 to 72 hr following oviposition. Stimulus intensity is expressed as the flux of LA, in moles/sec, passing over the mosquito antenna per unit time. The electrophysiological response is the change in nerve impulse frequency (ΔF) upon chemical stimulstion. It should be noted that the curve marked with the * was obtained from a female that still had eggs present even though she had been given the opportunity to oviposit. This curve is within the range of response functions for LA-excited neurons of blood-fed females prior to oviposition.

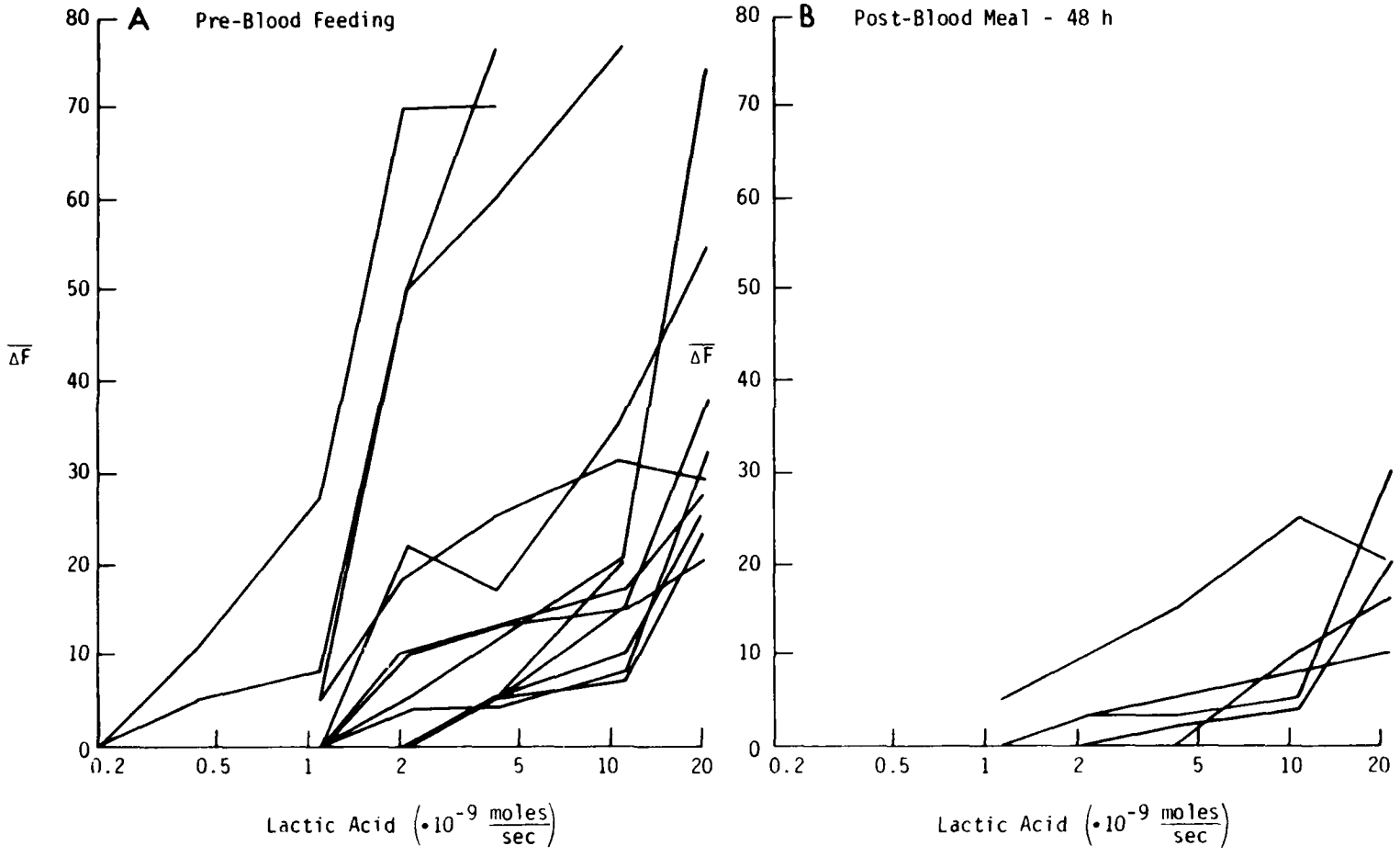
Fig. 2 Stimulus intensity vs. response functions of the oviposition attractant-sensitive neurons to methyl butyrate obtained from females fed on sucrose only (A) and from females 48 and 72 hr PBM (B). Part C is a comparison of the data in parts A and B. The hatched areas consist of the mean responses ± standard deviation for all nonblood-fed and 48 hr PBM females. The coordinates are the same as in Fig. 1 except that the stimulus is methyl butyrate.

Fig. 3 Stimulus intensity vs. response functions of the LA-excited neurons to LA obtained from female mosquitoes that were fed only on sucrose and that were injected with 0.5 µl of hemolymph drawn from (A) nonblood-fed females and (B) blood-fed females 48 hr PBM. The coordinates are the same as in Fig. 1.


A Pre-Blood Fed (n-11)
B Post-Blood Meal
24 h (n=3)
48 h (n=5)
72 h (n=4)
96 h (n=2)
C Post-Oviposition
$\Delta\bar{F}$ $(\frac{imp}{sec})$
Lactic Acid $(\cdot 10^{-9} \frac{moles}{sec})$
0
10
20
30
40
50
60
70
80
1
2
5
10
20
50


A
Non-Blood Fed Females
(n=17)
B
Blood Fed Females
48 h PBM (n=8)
72 h PBM (n=5)
ΔF
Methyl Butyrate (•10⁻⁷ moles/sec)


C
Short, pointed A2
Comparison of A and B
Pre-Blood Meal
48 h PBM
ΔF
Methyl Butyrate (•10⁻⁷ moles/sec)


A
Pre-Blood Feeding
B
Post-Blood Meal - 48 h
80
70
60
50
40
30
20
10
0
ΔF
0.2
0.5
1
2
5
10
20
Lactic Acid (•10^-9 moles/sec)

Structure-Activity Relationship of Lactic Acid-Excited Afferent Neurons in the Antennal Grooved-Peg Sensilla of the Mosquito *Aedes aegypti*

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Running title: Mosquito Lactic Acid Receptor

SUMMARY

The lactic acid (LA)-excited afferent neurons associated with the antennal grooved-peg sensilla of the mosquito were presented with a series of chemical stimuli selected to determine the structure-activity relationships of the LA receptor. The responses of single LA-excited neurons indicated that the optimal stimulus configuration for these neurons was a 3 carbon, α-hydroxy, monocarboxylic acid--i.e., LA. The range of sensitivities found in the sample of LA-excited neurons is discussed in relation to a potential mechanism for enhancing the overall sensitivity of the system to chemical stimuli. The efficiency of the grooved-peg sensilla as an olfactory receptor does not appear to be affected by the fact that it has only a single terminal pore. The electrophysiological data are correlated, insofar as possible, with behavioral data from the literature.

Introduction

Most animals, including man, produce lactic acid (LA) during normal metabolic activity. Some of the LA finds its way into the air surrounding these animals either by being excreted in the sweat, where it can then evaporate, or by being exhaled in the breath. Several investigators have demonstrated that female mosquitoes are attracted to the source of an air flow that contains LA and a small amount of carbon dioxide (Acree *et al.*, 1968; Smith *et al.*, 1970). Davis and Sokolove (1976) reported finding two types of LA-sensitive neurons associated with the grooved-peg sensilla on the antennae of the yellow fever mosquito. One type showed an increase in spike discharge rate on exposure to LA, whereas the second type showed a decrease in spike frequency in the presence of LA. Furthermore, they found that the behavioral synergism of LA plus CO_2 reported by Smith *et al.* (1970) was most likely the result of the summation of sensory information in the CNS and was not due to the interaction of LA and CO_2 at the primary receptor level. In a preliminary study, I reported that the spectrum of airborne stimuli to which these neurons would respond was limited to substances closely related to LA (Davis, 1977).

The purpose of the investigations reported herein was to extend our preliminary efforts toward defining the specificity and the sensitivity of the LA-excited antennal neurons to airborne chemical stimuli. By correlating the responsiveness of the LA-excited neurons to a series of stimuli whose molecular configurations were systematically varied, we were able to describe the structure-activity relationship of these neurons. Our electrophysiological data are compared with behavioral data obtained using many of the same stimuli reported in the literature.

Materials and Methods

The general procedures used in this study have been described elsewhere (Davis and Sokolove, 1976). Briefly, they are as follows.

Experimental Set-up. *Aedes aegypti* mosquitoes reared in our laboratory were anesthetized with CO_2, mounted on a holder, and placed under a microscope.

An uninsulated tungsten microelectrode (< 1 μ tip diameter) was inserted into the hemolymph space at the tip of the antenna and connected to ground. A similar electrode was inserted through the cuticle at the base of a sensillum and connected to various electronic devices to detect, amplify, and record sensory afferent nerve action potentials (nerve impulses or spikes). The nerve spikes were also routed to a three-channel amplitude discriminator, the output of which was connected to a frequency converter for plotting the instantaneous (spike-to-spike) frequencies of up to three individual sensory neurons.

In addition, the impulses, along with a stimulus marker, were photographed with a Grass C-4 camera. The films were then examined for evidence of spikes from more than one neuron that otherwise may not have been discriminable.

Chemical Stimulus Delivery. Chemical stimuli were presented in two ways. Initially, the responses of the sensory neurons were examined for their qualitative effects only. In these experiments, stimuli were generated by placing a 1.27-cm-diameter circle of filter paper saturated with a test substance in a 10-ml syringe. The air in the syringe was manually expelled slowly over the mosquito's antenna. The substances presented in this fashion are listed in Table 1. All chemical stimuli were obtained as either reagent or chromatographic grade compounds from Sigma Chemical, Aldrich Chemical, or Chemical Company.

The second method of stimulus generation was used to determine the stimulus intensity (dose)-response characteristics of the neurons. Air was passed through a flask containing the test liquid. This saturated the air stream at the vapor pressure of the test substance. One of two air streams was saturated in this manner; the other was left "clean." The flow rates of the two air streams were controlled by individual flowmeters and metering valves. By varying the ratio between the air streams, we could vary the intensity of the chemical stimuli.

Stimulation of the mosquito was accomplished by activating a solenoid valve that routed the air streams through a common delivery tube held near the mosquito's antenna. The chemical stimuli presented in this manner are indicated by the asterisk in Table 1.

Data Reduction. Instantaneous frequency records were scanned by eye and measured by hand at various times in each stimulus interval. Specifically, we measured the prestimulus or spontaneous tonic activity, the initial phasic response to a stimulus, and the later tonic response. In those cases where a cell had a highly variable discharge rate, its average tonic frequency could usually be estimated to within 5 impulses/sec at worst; the peak frequency, in most cases, could be measured to within 2 impulses/sec.

Results

An example of both types of LA-sensitive neurons associated with the grooved-peg sensilla on the antennae of adult female *Aedes aegypti* mosquitoes is presented in Fig. 1. The experimental results presented here were obtained from 106 grooved-peg sensilla that exhibited an increase in afferent spike activity on exposure to lactic acid. Responses of the LA-inhibited afferent neurons are not included in the work described here.

The first set of experiments was designed to provide a qualitative estimate of which chemical stimuli would evoke a response from the LA-excited neurons. The stimuli used were 2-, 3-, and 4-carbon analogs of lactic acid that had been selected to systematically test the importance of a particular chemical moiety, the importance of its position, or the number of such reactive groups. The results are presented in Table I. Certain substances that evoked relatively strong responses from the LA-excited neuron were selected for subsequent examination at lower stimulus intensities.

In examining the chemical structures vs. the responses elicited by the compounds in Table I, it becomes apparent that the 2- and 4-carbon analogs--glycolic and α-hydroxy-butyric acids--were less effective stimuli than LA. Of the 3-carbon compounds, those with a non acid terminal group--glycerol, isopropanol, glyceraldehyde--or with the terminal carboxyl group masked by an ester linkage--ethyl lactate--were relatively ineffective stimuli. The unsaturated compounds--acrylic and propionic acids--were very effective stimuli neat but less effective at lower intensities, as discussed later. Changing the hydroxyl group from the α- to the β-carbon--β-hydroxylpropionic acid--similarly reduced the effectiveness. Substitution for the α-hydroxyl of lactic acid by -SH, -Br, $-NH_2$, =O, $-CH_3$, or -H produced responses of varying degrees of effectiveness from strong (-SH, =O, and -Br) to weak ($-CH_3$ and -H). Malonic acid, with a carboxyl group at each end of the molecule and an α-OH, was not an effective stimulus. The inclusion of a second -OH group on the β-carbon--glyceric acid--was only slightly less effective than in LA eliciting a response from the LA-excited neurons.

Those chemical stimuli that elicited a relatively strong response from the LA-excited neurons when used neat (previous experiment) were subsequently tested at lower, more physiological intensity levels. The substances selected

were α-alanine and propionic, pyruvic, thiolactic, glyceric, acrylic, and α-bromopropionic acids. Chemical stimuli were generated using the system in which the intensity is calculated from vapor pressure temperature and flow rate (the second method in Materials and Methods). The stimulus intensities indicated were checked for accuracy by gas chromatographic analysis of samples from the various stimulus air streams collected in a liquid-nitrogen cold trap. The stimulus intensity-response curves in Fig. 2 were obtained from a group of LA-excited neurons to which we were able to present two or more stimuli to a single sensillum. The relative positions of the clusters of stimulus intensity-response curves for each of the three α-substituted analogs along the stimulus intensity axis indicates the order of effectiveness of the -OH, -SH and halide (Br) substitutions in eliciting an excitatory neural response. 2 & 3

Potential differences between the responsiveness of the LA-excited neurons to the D(-)- and L(+)-isomers of LA were determined by presenting each isomer in a graded intensity series and noting any differential changes in spike frequency between the two isomers. The responses of the LA-excited afferent neurons to D(-)-LA were not significantly different from those elicited by L(+)-LA (Fig. 3).

Discussion

Receptor specificity (selectivity). Two primary functions of any receptor system are the recognition of an appropriate signal and the mediation of a biological response. It is common practice to use the mediation of the biological response as a measure of the capability of the receptor to perform the recognition function. In chemoreceptor systems, the structure-activity relationship (SAR) provides the best measure of how selective the receptor is in recognizing the appropriate stimulus ligand. Through the use of a carefully selected series of analog compounds, it is possible to determine the

optimum stimulus configuration for th receptor in question as well as other properties of the receptor-ligand int ractions. From our SAR analysis of the LA-excited neuron, we conclude that the optimum configuration for a compound to elicit a biological response, as measured by a change in the spike discharge pattern, from these afferent neurons should have the following features:

- Three-carbon chain length--

  Lactic acid >> glycolic acid ≥ α-hydroxybutyric acid
  ($CH_3CHOHCOOH$) ($CH_2OHCOOH$) ($CH_3CH_2CHOHCOOH$)

- Mono-carboxylic acid--

  Glyceric acid >> isopropanol ≥ glyceraldehyde, glycerol,
  ($CH_2OHCHOHCOOH$) ($CH_3CHOHCH_3$) ($CH_2OHCHOHCHO$) ($CH_2OHCHOHCH_2OH$)

  and malonic acid > ethyl lactate
  ($HOOCCHOHCOOH$) ($CH_3CHOHCOOCH_2CH_3$)

- Side-group in the α position--

  Lactic acid > propionic acid >> β-hydroxypropionic acid
  (CH_3CH_2COOH) (CH_2OHCH_2COOH)

- α-Side group not rigidly specific--

  -OH ≥ -SH > -Br = -Cl >> $-NH_2$, $-CH_3$,
  lactic acid; thiolactic acid; α-bromo- and α-chloro-propionic acid; α-alanine; isobutyric acid

  -H, =O
  propionic acid; pyruvic acid

- Other--

  α-Substitution in addition to -OH--less effective than -OH alone

  Lactic acid > α-Hydroxyisobutyric acid
  [$(CH_3)_2COHCOOH$]

  Unsaturated site--less effective than certain α-substitutions (at physiological levels)

  Lactic acid > acrylic acid ≯ propiolic acid
  ($CH_2=CHCOOH$) ($CH \equiv CCOOH$)

The data in Table I indicate that the receptor did not appear to be rigidly specific for any one of the side-group substitutions on the α-carbon. A comparison of the quantitative responsiveness of the most likely candidates from that series of compounds indicates that the hydroxyl group in the α-position was most effective in eliciting a biological response (Fig. 2).

Comparison of the responses of the LA-excited neurons to D(-)- and L(+)-LA indicates that the receptor does not differentiate between these two isomers--at least over the intensity range tested. Kafka *et al.* (1973) reported a similar lack of specificity for steric conformation in one of two neurons sensitive to 4-methyl-hexanoic acid in *Locusta migratoria*. Receptors on the other neuron did, however, differentiate between the (-)- and (+)-enantiomers of 4-methyl-hexanoic acid. LA has two primary reactive groups--i.e., the α-hydroxyl and the carboxyl groups--and may react with the receptor on the LA-excited neuron in a manner similar to the reaction of LA with the enzyme, lactate dehydrogenase--i.e., only two points of interaction between substrate and enzyme--(Baker, 1967). If this is the case, the critical features of the two isomers would appear nearly identical and the receptor would not differentiate between them.

From these SAR data we conclude that lactic acid is the stimulus ligand that the receptor on the LA-excited neurons is most capable of recognizing but that other compounds at appropriate intensities may also generate a biological response in these neurons. Whether the responses evoked by other stimuli are qualitatively differentiable is dependent on properties of the neural code intrinsic to these neurons (O'Connell, 1975; Perkel and Bullock, 1968).

Receptor sensitivity. A cursory examination of the stimulus-response curves of Fig. 2 suggest that there is considerable variability in the relative responsiveness of the LA-excited neurons to LA. This variability could be attributed to such physical factors external to the sensilla as differences in stimulus and/or ambient airflow rates, or position of the sensillum on the antenna relative to the odor airstream. Factors internal to the mosquito, such as age or physiological state, also might affect the response. However, in any

single experiment, the external factors and the age of the mosquito are relatively constant and hence can be excluded as the source of variability. It should be noted that, the degree of variability observed between sensilla on a single antenna is undifferentiable from that observed between sensilla on different antennae, and that the response variation between sensilla does not occur in any systematic manner. O'Connell (1975) has discussed response variability resulting from some uncontrolled physiological changes in the insect that alter the general level of excitability of an individual neuron on a moment-to-moment basis. His argument for differences observed between two neurons within a sensillum can be extended to include similar differences between two neurons in different sensilla and, for that matter, in different mosquitoes. Briefly, if two receptor neurons give nearly identical responses to an intensity series of a compound, then it can be assumed--provided that all other factors are equal--that the two receptors are nearly identical. However, any deviation between the responses of the two neurons to a second compound indicates that the receptors must have absolute differences in the kind, number, or distribution of receptor sites on their respective membranes. As this description fits the response characteristics of the LA-excited neurons, the apparent variability between responsiveness across this sample of LA-excited neurons represents real differences in their intrinsic membrane receptor properties. Thus, what at first appears as variability in response may in fact represent a means by which the mosquito has increased its capability of odor discrimination over a wide range of stimulus intensities. This can be explained as follows. If a population of chemoreceptor neurons had nearly identical sensitivities to chemical stimuli--the notion of a "typical" or "normal" type receptor--then any single neuron would have to encode intensity information over the entire range of stimulus intensities to which the insect may be expected to respond. As this information is provided in the form of changes in the spike discharge pattern or frequency, small incremental changes in odor intensity would produce only small changes in

spike activity. Furthermore, any noise in the system in the formation irregular spike frequency pattern would result in a high error rate in the CNS when attempts are made to distinguish small changes in odor intensity. In contrast, if the system were made up of units, each of which had its own stimulus intensity-response characteristics over a narrow portion of the stimulus intensity range for a compound, and the stimulus intensity-response characteristics for the population spanned the entire intensity range over which the insect might respond to that compound, then--taken collectively--they could provide a high degree of odor intensity discrimination. Small changes in odor intensity would be reflected by large changes in the spike discharge frequency in only those neurons that were active within that portion of the intensity range. A system of this type would be more sensitive to the small differences in local odor intensity in an airflow emanating from a potential host and less sensitive to 'noise' than would a system comprised of cells having nearly identical stimulus intensity-response characteristics.

Morphological considerations. McIver (1974) described the antennal grooved-peg sensillum of the mosquito as being approximately 7.8 to 10.4 μm long and having a single terminal pore. Most sensilla with a single terminal pore are found on areas of the insect anatomy where they may be employed as gustatory or contact chemosensory organs (Zacharuk, 1980). However, the antennal grooved-peg sensillum is clearly an olfactory receptor for these reasons. First it responds to chemical stimuli in the vapor phase at lower stimulus intensities than it does to aqueous stimuli (Davis, unpublished observations). Second, because it is short, it is situated well inside the envelope of longer chemo- and mechano-sensory antennal hairs and hence cannot come into contact with the substrate. Third, the mosquito does not use its antenna as a contact chemosensory organ.*

*Exceptions, as always, do exist--see Provost and Haeger (1967)

Therefore, even though the grooved-peg sensillum fits the description of contact chemoreceptor sensilla, it is clearly an organ for detecting airborne chemical signals.

The fact that the grooved-peg sensillum possesses only a single terminal pore would lead one to think that it might be less efficient in allowing odor molecules access to the LA-sensitive neurons within, than sensilla that have numerous pores along their lengths. This does not seem to be the case, as odor intensities on the order of 10^{-9} moles/sec are sufficient to evoke changes in the neural activity of the neurons in the sensilla. This stimulus flux level is approximately equivalent to an odor airstream containing 10^{-12} moles/ml. These levels are comparable to the levels reported by Kafka *et al.* (1973) for *Locusta migratoria*. The grooved-peg sensillum appears to be as efficient an olfactory organ as its multiporous counterparts.

Behavioral correlations. One of the objectives of these experiments was to correlate our electrophysiological findings with behavioral observations reported in the literature (Carlson *et al.*, 1973). The two sets of data agree reasonably well. For example, for the compounds selected to determine the specificity of the insects for the carboxyl group, the ratio of mosquitoes attracted by the test chemical to the number attracted by LA resulted in the following rank ordering (ratios in parentheses): LA > glyceric acid (0.3) > glyceraldehyde (0.2) $\geq$ glycerol (0.1) = malonic acid (0.1). A similar ranking was observed in the electrophysiological data. In a few cases, however, the correlation was weak or nonexistent, probably due to the differences in methodology. Carlson *et al.* (1973) compared the substances at equal weights, whereas we took temperature and vapor pressure into consideration when selecting stimulus intensities. This is an important difference, because some

of the compounds differ markedly in their vapor pressures, e.g., the V_p (25°C) for glycerol is 0.05 mm Hg, whereas that for propionic acid is 4.0 mm Hg (Weast, 1977).

Acknowledgements

I wish to thank Dr. Ron Spanggord for GC analysis of the odor airstreams and Dr. Nancy Rachman for her critical review of the manuscript. This research was supported by NIH Grant AI-10954 and U.S. Army Medical Research and Development Command Contract DAMD-17-78C-8022.

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Figure Legends:

Fig. 1. Spike potentials from two lactic acid-sensitive neurons; one excited and one inhibited by lactic acid. Lower line in each set is the stimulus mark.

Fig. 2. Stimulus intensity vs response for a series of LA-excited neurons: Upper--to LA (N = 17); Middle--to thiolactic acid (N = 5); Lower--to 2-bromopropionic acid (N = 10). The stimulus intensity (S) scales are identical in each group of curves and have been vertically aligned for comparison of the groups of curves. The response is the change in spike frequency (ΔF), in pulses/sec (pps) elicited by the stimulus ($F_{stim} - F_{spontaneous}$).

Fig. 3. Comparison of the stimulus intensity vs response curves for four LA-excited neurons exposed to both D(-)- (broken line) and L(+)-LA (solid line). The response is the change in spike frequency (ΔF) on stimulation.

Table

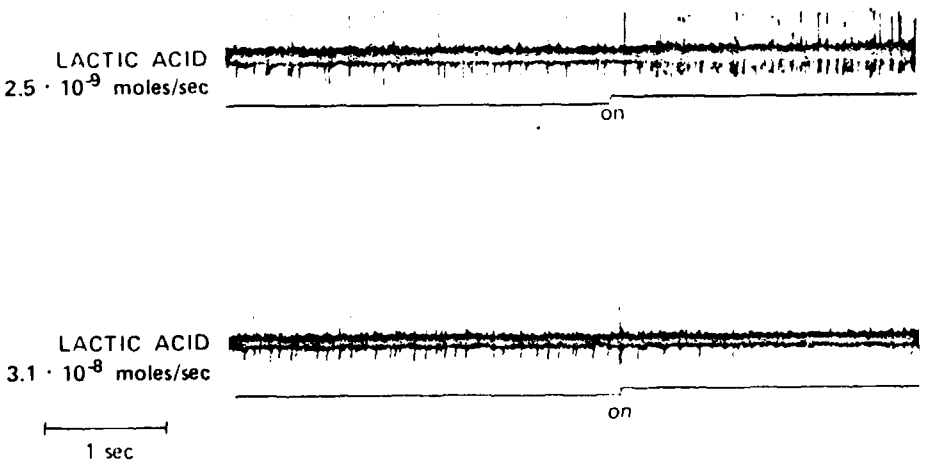
QUALITATIVE RESPONSES OF LACTIC ACID-EXCITED CELLS TO LACTIC ACID RELATED COMPOUNDS

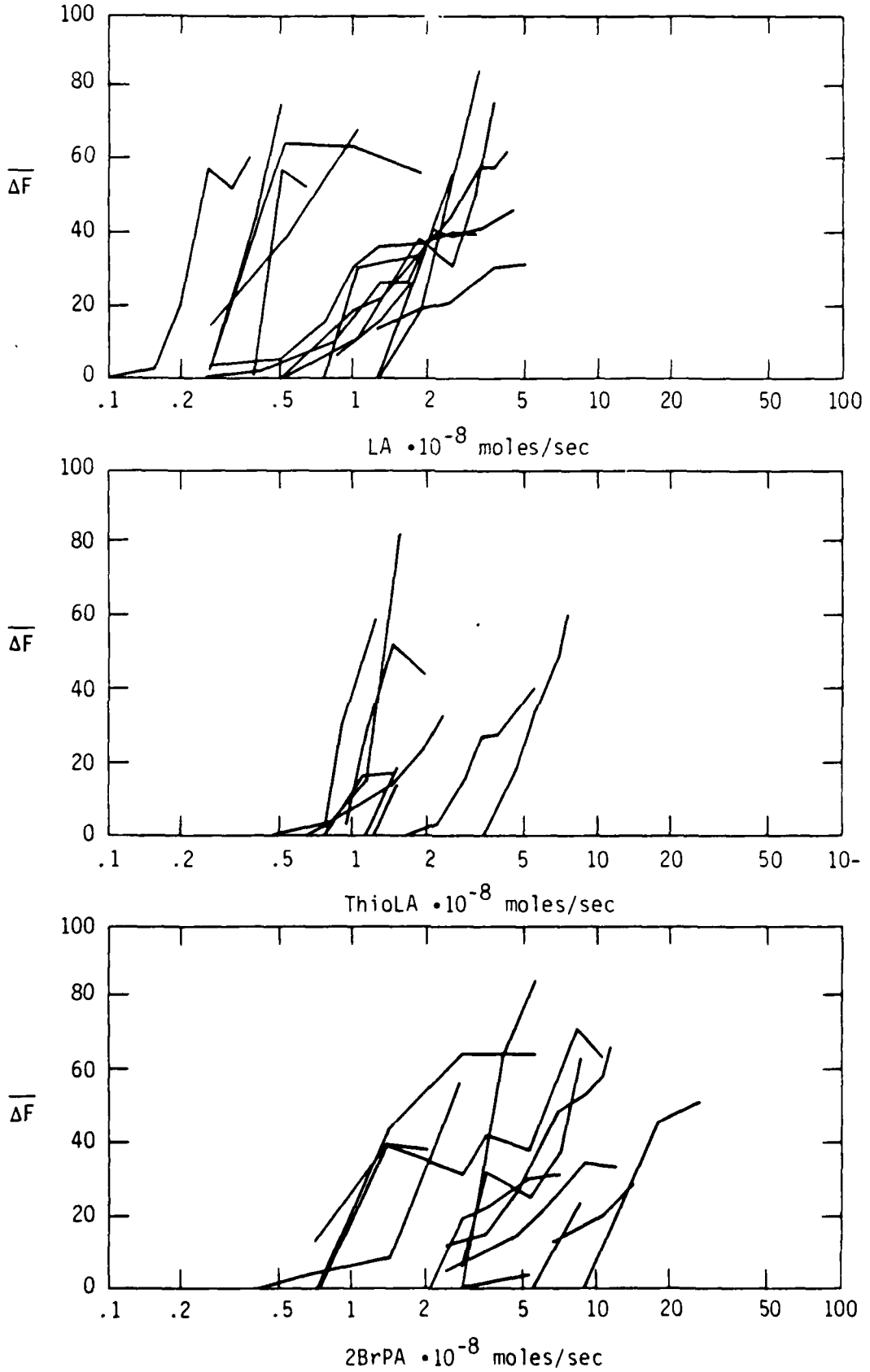
Lactic Acid Related Compounds[b]	Structure	Response (Change in Spike Frequency)				
		Increase, Strong	Moderate	Weak	No Change	Decrease
2-Carbon:						
Acetic Acid	CH_3COOH	3[a]	1			1
Glycolic Acid	$HOCH_2COOH$	1	6	10	15	
Oxalic Acid	$HOOCCOOH$				4	
Oxamic Acid	$H_2NCOCOOH$			1	3	
3-Carbon						
* Propionic Acid	CH_3CH_2COOH	9	4	1	9	7
* Pyruvic Acid	$CH_3COCOOH$	13	3	1	7	3
* Thiolactic Acid	$CH_3CH(SH)COOH$	16	14	6	11	2
* α-Bromo-Propionic Acid	$CH_3CH(Br)COOH$	25	3	1	9	2
* α-Chloro-Propionic Acid	$CH_3CH(Cl)COOH$	13	1	1	2	3
* α-Alanine	$CH_3CH(NH_2)COOH$	6	3	5	12	
Isopropanol	$CH_3CH(OH)CH_3$	4		17		1
Malonic Acid	$HOOCCH(OH)COOH$	2	1	5	12	
β-Hydroxy-Propionic Acid	$HOCH_2CH_2COOH$	6	1	3	23	2
Glycerol	$HOCH_2CH(OH)CH_2OH$		1		19	
* Glyceric Acid	$HOCH_2CH(OH)COOH$	5	3	2	7	
Glyceraldehyde	$HOCH_2CH(OH)CHO$			1	14	
* Acrylic Acid	$CH_2=CHCOOH$	12	3	1	1	2
Propiolic Acid	$CH\equiv CCOOH$	1				
4-Carbon:						
Butyric Acid	$CH_3CH_2CH_2COOH$	2	1	1	1	6
α-Hydroxy-Butyric Acid	$CH_3CH_2CH(OH)COOH$		3	13	19	
Isobutyric Acid	$(CH_3)_2CHCOOH$	5	3	1	2	7
α-Hydroxy-Isobutyric Acid	$(CH_3)_2C(OH)COOH$		2		4	
Other:						
Ethyl-lactate	$CH_3CH(OH)COOC_2H_5$				3	1

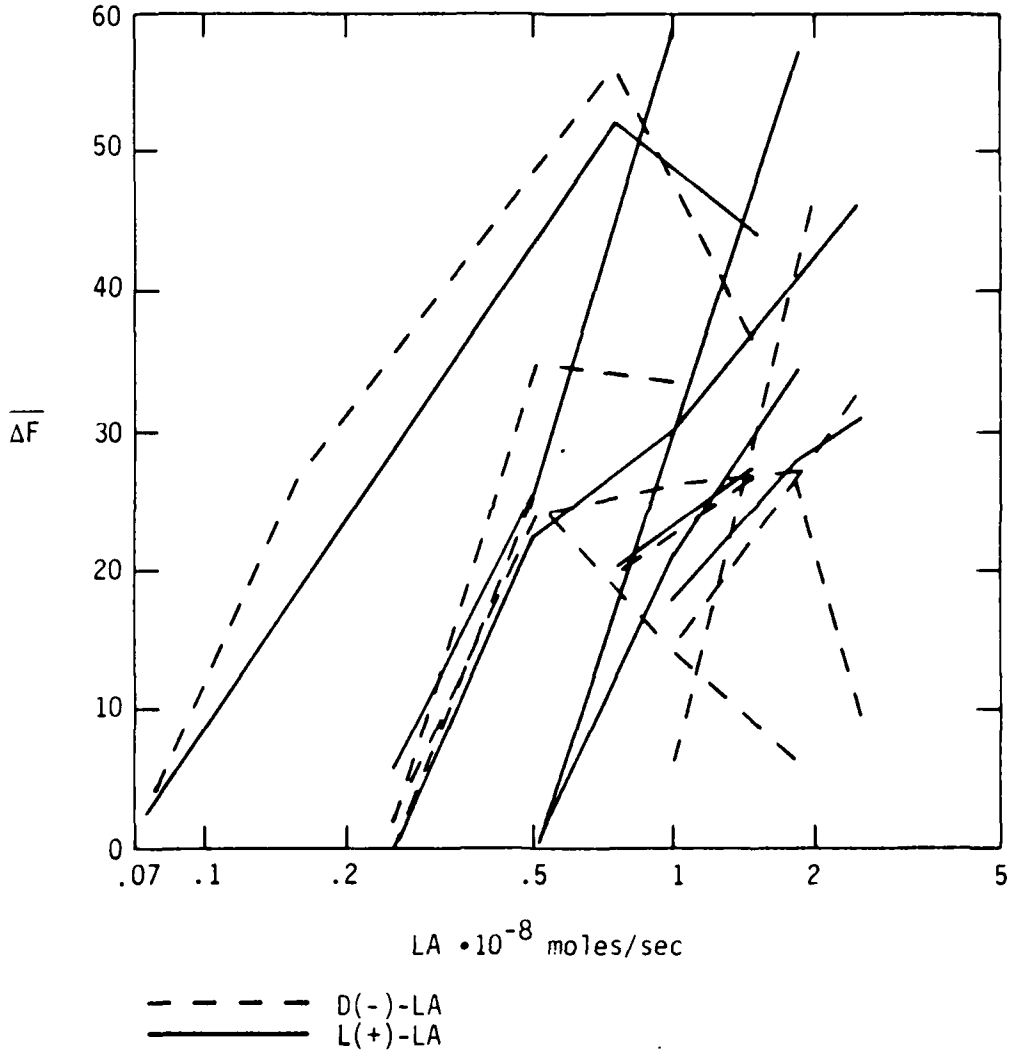
Note (a) Number of cells exhibiting the indicated relative changes in their spontaneous discharge rates

(b) All liquids used neat. All solids used as saturated aqueous solutions

GROOVED-PEG SENSILLA
Aedes aegypti (♀)




100
80
60
40
20
0
ΔF
.1
.2
.5
1
2
5
10
20
50
100
LA •10^-8 moles/sec
ThioLA •10^-8 moles/sec
2BrPA •10^-8 moles/sec


60
50
40
30
20
10
0
$\overline{\Delta F}$
.07
.1
.2
.5
1
2
5
LA $\cdot 10^{-8}$ moles/sec
D(-)-LA
L(+)-LA

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α-position was most effective in eliciting a biological response (Fig. 2).

END

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